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(54) Title: MODIFIED PEPTIDES AND THEIR USES

(57) Abstract: A modified pro-α chain comprising a triple helix forming domain linked to at least an N-terminal domain, the N-terminal domain containing a polypeptide from at least part of a proteoglycan core, such as decorin. The pro-α chain may form part of a procollagen molecule that has the N-terminal domain retained. The procollagen molecules may be incorporated into collagen polymers, matrices and gels and be used for such applications as wound healing, tissue replacement and cosmetic treatments.

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Title: MODIFIED PEPTIDES AND THEIR USES

DESCRIPTION

The present invention relates to modified extracellular matrix molecules, to polymers, matrices and gels made therefrom and to their uses in such applications as wound healing and cosmetic treatments.

Most cells, whether simple unicellular organisms or cells from human tissue, are surrounded by an intricate network of macromolecules which is known as the extracellular matrix (ECM) and which is comprised of a variety of proteins and polysaccharides.

A major protein component of the ECM is a family of related proteins called the collagens which are thought to constitute approximately 25% of total proteins in mammals. There are at least 26 genetically distinct types of collagen molecule, some of which are known as fibrillar collagens (collagen types I, II, III, V and XI) because they typically form large fibres, known as collagen fibrils, that may be many micrometers long and may be visualised by electron microscopy.

Collagen fibrils are comprised of polymers of collagen molecules and are produced by a process involving conversion of procollagen to collagen molecules that then assemble to form the polymer. Procollagen consists of a triple stranded helical domain in the centre of the molecule and has non-helical domains at the amino terminal (known as the N-terminal propeptide) and at the carboxyl terminal (known as the C-terminal propeptide). The triple stranded helical domain is made up of three polypeptides which are known as α chains. Procollagen is made intracellularly from pro-α chains (α chains with N and C-terminal forming propeptides domains). Pro-α chains are synthesised on membrane-bound ribosomes following which the pro-α

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chains are inserted into the endoplasmic reticulum. Within the endoplasmic reticulum the pro-α chains are assembled into a procollagen molecule. Procollagen is secreted into the extracellular environment where it is then converted into collagen by the action of procollagen N-proteinases (which cleave the N-terminal propeptide) and procollagen C-proteinases (which cleave the C-terminal propeptide). Once the propeptides have been removed the collagen molecules thus formed are able to self-assemble spontaneously to form the collagen fibrils. The rate determining step in the formation of collagen fibrils is the removal of the C-propeptides by procollagen C-proteinases.

Collagen fibrils interact with other fibrils and also other components of the extracellular matrix to form connective tissues *in vivo*. Fibrils will assemble *in vitro* and will interact to form a collagen matrix or gel. Such collagen matrices have various industrial uses. For instance, collagen-based biomedical products are used in the cosmetic and aesthetic enhancement markets as implants and for smoothing lines, wrinkles and facial scars. Collagen based products are also used in the production of artificial skins (e.g. for treating burns patients), wound dressings and the like.

Whilst collagen based products have been extensively adopted, their performance is far from satisfactory and a number of contra-indications and adverse reactions are known. Some of the problems are related to the fact that many of these products are based on animal collagen (e.g. from bovine hide) and as such can give rise to allergic and inflammatory reactions and infections. Other collagen products are derived from cadaver tissue and it is suggested that they result in reduced inflammation and allergic reactions. However such products are expensive to manufacture and difficulties in controlling product quality can lead to variation in performance.

Another problem associated with known artificial collagen matrices or gels is that they tend to contract over time. Gel contraction can be a particular problem when artificial collagen matrices or gels are exposed to living cells (*in vitro* or *in vivo*). This phenomenon is highly undesirable especially when collagens are used as wound dressings or in artificial skin.

Another important function of the ECM is the storage and presentation of growth factors to cells. Proteoglycan components of the ECM play a central role in the regulation of the activity of a number of growth factors and therefore represent powerful pathophysiological modulators.

A well known example of a family of proteoglycans has a core protein of about 40 kDa that consists mainly of leucine-rich repeats of 20 - 24 amino acids. These proteins are known as Small Leucine-Rich Proteoglycans (SLRPs) and typically contain the sequence $LX_3LXLX_2NX(L/I)$ where L = leucine; N= asparagine are in the specified conserved positions and X = any amino acid.

The SLRP family comprises at least 4 members, namely decorin, biglycan, fibromodulin and lumican (all of which were characterised in some detail in the late 1980s/early 1990s). These proteoglycans have specialised functions in cell cycle regulation, in tissue repair and in modulating the mechanical properties of tissues by their interaction with collagen fibrils. Decorin and related proteoglycans have also been reported to bind to and modulate the activity of various growth factors including members of the transforming growth factor β (TGF-β) family. Growth factors such as the TGF-βs have a major influence on cell activity and ECM remodelling. There are at least 5 different TGF-βs (TGF-β1 - TGF-β5) and their chemical structures and activity have been widely reported (e.g. see Sporn *et al.* J. Cell Biol. 105: 1039 (1987).

A major pathophysiological activity of TGF-βs (particularly TGF-β1 and TGF-β2) is the promotion of wound healing. However this is often associated with increased scar formation and fibrosis. In fact, clinical interest in the modulation of TGF-β has been associated with inhibiting its activity in order to reduce scar formation (although this may compromise the rate of wound healing). For instance, WO 92/17206 discloses compositions which inhibit the activity of TGF-β1 and TGF-β2 and are particularly beneficial for reducing scar formation.

Another proteoglycan that is known to bind to TGF-βs is the type III TGF-β receptor. This proteoglycan is a cell membrane receptor that can act as a reservoir for TGF-β and is also known as betaglycan (or soluble betaglycan if cleaved from the cell membrane and found free in the ECM).

The modulation of the activity of growth factors such as TGF-β is of significant clinical interest. Various parties have investigated the usefulness of proteoglycans as pharmacologically active agents. For instance, the use of such molecules to regulate fibrotic conditions, wound healing and scarring is contemplated in:

(1) WO 93/09800 - relating to the use of decorin and related proteoglycans as agents for preventing or reducing scarring; and

(2) WO 97/05892 - which discloses the use of soluble betaglycan as an anti-scarring agent.

Despite these advances there remains a need to develop improved medicaments for regulating growth factor activity.

In particular there is a need to develop a system that may be used to deliver growth factors to a tissue of interest (e.g. a wound site or site of fibrosis) and sensitively regulate growth factor activity in that tissue.

It is an object of the present invention to address problems associated with prior art medicaments and delivery systems. A further object of the present invention is to address problems associated with collagen matrices and gels known to the art.

The present invention is based upon the realisation by the inventors that desirable functional characteristics may be introduced into a composition such as a medicament or collagen matrix by designing modified pro-α chains according to a first aspect of the present invention which may be trimerised to form procollagen

derivatives. These in turn may be converted to collagen monomers (with retained propeptides) and subsequently polymerised. This allows the synthesis and assembly of novel collagen polymers having new biological properties.

To this end, a first aspect of the present invention provides a modified pro-α chain comprising a triple helical forming domain linked to at least an N-terminal domain characterised in that the N-terminal domain contains a polypeptide sequence from at least part of a proteoglycan core.

The inventors have found that they can employ molecular biology techniques to modify the gene encoding pro-α chains such that modified pro-α chains according to the first aspect of the invention may be expressed therefrom. Therefore according to a second aspect of the invention there is provided a DNA molecule encoding modified pro-α chains according to the first aspect of the invention.

The inventors then trimerised modified pro-α chains according to the first aspect of the invention to form a procollagen molecule with a modified N propeptide. The trimer may be a homotrimer of modified pro-α chains or may be a heterotrimer also containing natural pro-α chains. Therefore according to a third aspect of the present invention there is provided a procollagen molecule comprising a trimer of pro-α chains characterised in that at least one of the pro-α chains is a pro-α chain according to the first aspect of the invention.

The inventors then performed further experiments that established that procollagen molecules according to the third aspect of the invention may be polymerised to form a collagen polymer. Furthermore they have established that they can regulate N-propeptide cleavage by modifying the N-terminal domain such that the domain's susceptibility to cleavage is altered such that the collagen polymer retains N-propeptides or derivatives thereof upon its surface. This may be achieved by designing procollagen molecules according to the third aspect of the invention such

that they are resistant to procollagen N-proteinases. Alternatively, the molecules may only be partially cleaved or cleaved more slowly. It is preferred that pro-α chains according to the first aspect of the invention are also modified such that they contain an amino acid sequence that confers resistance to procollagen N-proteinases.

Alternatively the inventors have found that they can assemble collagen polymers with retained N-propeptides in an environment in which procollagen N-proteinase is either inhibited or absent.

According to a fourth aspect of the invention there is provided a collagen polymer with at least some of the collagen monomers contained therein having retained N-terminal ends characterised in that at least some of the retained N-terminal ends contain a polypeptide sequence encoding at least part of a proteoglycan protein core.

Collagen polymers according to the fourth aspect of the invention may form collagen fibrils.

Additionally, the C-terminal domains of the procollagens making up the collagen polymer are preferably removed, for example using a procollagen C-proteinase, such as bone morphogenetic protein (BMP-1). This has been found to result in the N-terminal propeptides being presented at the fibril surface.

EP-A-0 985 732 contemplates the production of chimeric collagens with biologically active peptides (e.g. a growth factor *per se*) fused to the N-terminal and which can polymerise to form fibrils. However EP-A-0 985 732 does not contemplate or suggest the addition of a proteoglycan to the N terminal domain of a pro-α chain according to the first aspect of the invention. Furthermore there is no suggestion in EP-A-0 985 732 that an N modified procollagen may be produced with the kind of surprising beneficial effects that are outlined in more detail below.

Modified pro-α chains according to the first aspect of the invention are preferably modified forms of fibrillar forming procollagens (e.g. modified forms of

type I, II, III, V or XI pro-α chains). Preferably the molecule is a modified type III pro-α chain. This type is preferred because it can co-assemble with type I collagen and can also form a homotrimer. It is most preferably a modified proα1(III) chain.

It is prefered that the N-terminal ends are derived from Small Leucine-Rich Proteoglycans (SLRPs) that have a core protein of about 40 kDa. It is preferred that the N-terminal domain is modified such that it comprises at least one leucine-rich repeat of 20 - 24 amino acids found in the decorin family of proteoglycans. It is most preferred that the N-terminal end comprises the amino acid sequence $LX_3LXLX_2NX(L/I)$ [where L = leucine; N= asparagine are in the specified conserved positions and X = any amino acid] and multiples thereof.

Natural N-terminal propeptide forming domains may be modified such that essentially all of the N-terminal end is replaced by a proteoglycan molecule (e.g. decorin, biglycan, fibromodulin, lumican, betaglycan and functional derivatives thereof). The extent to which the normal N-terminal propeptide forming domain is replaced is less critical than ensuring proteoglycan functionality is introduced. Accordingly the N-terminal propeptide forming domain may be totally replaced, partially replaced or even maintained in its entirety (provided it has added proteoglycan functionality).

It is desirable to make some modified pro-α chains according to the present invention that trimerise to form procollagens that are resistant to N propeptide cleavage. Therefore some preferred molecules according to the first aspect of the invention have amino acid sequences defining a modified N-proteinase cleavage site which renders procollagens resistant to such cleavage. People with the Ehlers Danlos syndrome type VII have mutations in a collagen gene which abolishes the N-proteinase cleavage site on the procollagen molecule. Therefore with knowledge of this mutation the region of the domain requiring such modification is easily identified.

The region between the helical forming domain and N-propeptide forming domain of the pro-α chain (the so called hinge domain) is most suitably modified to

confer resistance to N-proteinases. For instance, Pro-Gln at the cleavage site may be altered to Leu-Pro.

Modified pro-α chains according to the first aspect of the invention may be formed by direct chemical synthesis or by *in vitro* amino acid polymerization followed by protein folding and, if appropriate, glycosylation of the proteoglycan polypeptide sequence. However it is preferred that molecular biology techniques are used to design a DNA molecule according to the second aspect of the invention and express the modified pro-α chain in a cell or expression system containing such a DNA molecule.

The DNA molecule according to the second aspect of the invention may be formed by manipulating the bases encoding the N-terminal propeptide forming domains such that amino acids are added, substituted or deleted. It is preferred that a nucleotide sequence encoding decorin, biglycan, fibromodulin or lumican or functional derivatives thereof is inserted into the bases encoding the N propeptide forming domain. It is particularly preferred that a nucleotide sequence encoding decorin is inserted into the bases encoding the N propeptide forming domain.

Preferred modifications include the insertion of a nucleotide sequence encoding at least one leucine-rich repeat of 20 - 24 amino acids found in the decorin family of proteoglycans.

It is also preferred that the N propeptide forming domain of the DNA molecule is modified such that it contains coding sequences from betaglycan and related proteoglycans.

Alternatively the bases encoding an N propeptide forming domain of a natural pro-α chain may be completely excised and replaced with bases encoding leucine rich repeat peptides or proteoglycan core proteins.

According to a preferred embodiment of the invention, the DNA molecule may encode a C-propeptide domain and an α-chain of a pro-α chain and may have the

"natural" N-propeptide entirely replaced by a sequence encoding a leucine rich repeat peptide or a proteoglycan core protein (e.g. decorin).

As previously indicated it is desirable to make some pro-α chains, procollagens or collagen polymers according to the present invention resistant to N propeptide cleavage. Therefore some preferred DNA molecules according to the second aspect of the invention have DNA sequences encoding a modified N-proteinase cleavage site which alters the proteins expressed therefrom resistance to such cleavage. Preferably, the expressed proteins are resistant to cleavage. Alternatively, cleavage in the expressed protein may be partial or slower than in the un-modified protein. It is preferred that the region between the helical forming domain and N-propetide forming domain of the pro-α chain (the so called hinge domain) is mutated to confer resistance to N-proteinases. For instance, nucleotides encoding Pro-Gln at the cleavage site may be altered to nucleotides encoding Leu-Pro.

The DNA molecule may be incorporated within a suitable vector to form a recombinant vector. The vector may for example be a plasmid, cosmid or phage. Such vectors will frequently include one or more selectable markers to enable selection of cells transfected with the said vector and, preferably, to enable selection of cells harbouring the recombinant vectors that incorporate the DNA molecule according to the second aspect of the invention.

Standard molecular biology techniques may be used to construct vectors comprising DNA molecules according to the second aspect of the invention. Preferred constructs and expression systems are described in more detail in the Examples.

Vectors may be expression vectors and have regulatory sequences to drive expression of the DNA molecule. Vectors not including such regulatory sequences may also be used and are useful as cloning vectors for the purposes of replicating the DNA molecule. When such vectors are used the DNA molecule will ultimately be required to be transferred to a suitable expression vector which may be used for production of the procollagen derivative of the invention.

Replication of the DNA molecule in cloning vectors or expression of the protein product from recombinant expression vectors is performed within a suitable host cell. The DNA molecule may be incorporated within a vector within the host cell. Such host cells may be prokaryotic or eukaryotic. Eukaryotic hosts may include yeasts, insect and mammalian cells. Hosts used for expression of the protein encoded by the DNA molecule are ideally stably transformed, although the use of unstably transformed (transient) hosts is not precluded.

A preferred host cell is the HEK293 cell line and derivatives thereof.

The DNA molecule of the invention may also be incorporated in a transgene construct designed for expression in a transgenic plant or, preferably, animal. Transgenic animals which may be suitably formed for expression of such transgene constructs, include birds such as domestic fowl, amphibian species and fish species. The protein may be harvested from body fluids or other body products (such as eggs, where appropriate). Preferred transgenic animals are (non-human) mammals, particularly placental mammals. An expression product of the DNA molecule of the second aspect of the invention may be expressed in the mammary gland of such mammals and the expression product may subsequently be recovered from the milk. Ungulates, particularly economically important ungulates such as cattle, sheep, goats, water buffalo, camels and pigs are most suitable placental mammals for use as transgenic animals according to the invention. The generation and usefulness of such mammalian transgenic mammary expression systems is both generally, and in certain instances specifically, disclosed in WO-A-8800239 and WO-9005188.

It is preferred that the host contains suitable intracellular facilities for the assembly of the procollagen derivative of the first aspect of the invention from the protein products of the DNA molecule of the second aspect of the invention. In particular, expression hosts, particularly transgenic animals, may contain other exogenous DNA the expression of which facilitates the expression, assembly, secretion or other aspects of the biosynthesis of procollagen derivatives of the third aspect of the invention and even collagen polymers according to the fourth aspect of the invention. For example, expression hosts may co-express prolyl 4-hydroxylase,

which is a post translation enzyme important in the natural biosynthesis of procollagens, as disclosed in WO-9307889.

DNA, particularly cDNA, encoding natural pro-α chains is known and available in the art. For example, WO-A-9307889, WO-A-9416570 and the references cited in both of them give details. Such DNA may be used as a convenient starting point for making a DNA molecule of the present invention. Recombinant techniques may be used to derive the DNA molecule of the invention from such a starting point.

DNA sequences, cDNAs, full genomic sequences and minigenes (genomic sequences containing some, but not all, of the introns present in the full length gene) may be inserted by recombinant means into a DNA sequence coding for naturally occurring pro-α chains (such as the starting point DNA mentioned above) to form the DNA molecule according to the second aspect of the invention. Because of the large number of introns present in collagen genes in general, experimental practicalities will usually favour the use of cDNAs or, in some circumstances, minigenes. The inserted DNA sequences, cDNAs, full genomic sequences or minigenes code for amino acids which when expressed and assembled into a procollagen according to the third aspect of the invention give rise to a desired modification in the N-terminal domain of such a procollagen derivative.

Any of the DNA material used in these methods (including the DNA sequences, cDNAs, full genomic sequences and minigenes; the DNA molecule according to the second aspect of the invention and vectors) may be prepared by any convenient method involving coupling together successive nucleotides, and/or ligating oligo- and/or poly-nucleotides, including *in vitro* processes. However recombinant DNA technology forms the method of choice.

A preferred vector for DNA molecules according to the second aspect of the invention is the episomally replicating plasmid pCep4. This plasmid allows high levels of expression of cloned DNA molecules in cell-lines such as HEK293 transfected with the EBV nuclear antigen.

Collagen polymers in accordance with the fourth aspect of the invention may be of a number of forms. Cylindrical polymers similar to collagen fibrils are generated from mixtures of collagen molecules and collagens derived from procollagens according to the third aspect of the invention when collagen molecules are the major component. Alternatively, sheet-like structures may be formed by using procollagen derivatives according to the third aspect of the invention in the absence of, or substantially in the absence of, normal collagen molecules.

A remarkable feature of collagen polymers according to the fourth aspect of the invention is that the modified N-terminal propeptides are located to the surface of the polymer/fibril so formed, particularly in the case where the C-terminal domain of the procollagen has been removed. The inventors have demonstrated that fibrils formed from mixtures of natural collagens and modified procollagens according to the third aspect of the invention exhibit the proteoglycan N-propeptides at the fibril surface whereas the natural collagens (i.e. those without retained N-propeptides) form the core of the fibril. The arrangement of the molecules in the fibril optimises presentation of the N-propeptides to the interfibrillar space.

Additionally, the inventors were able to form collagen matrices from procollagen molecules according to the third aspect of the invention and/or collagen polymers according to the fourth aspect of the invention. Such matrices represent an important fifth aspect of the invention in that the matrices have surprisingly been found to be resistant to shrinkage.

Accordingly, a fifth aspect of the present invention provides a collagen matrix comprising some collagen monomers characterised in at least some of the monomers have modified N-terminal domains that provide a matrix that is resistant to contraction.

Preferably, the matrix is characterised by the fact that at least some of the collagen monomers have a N terminal domain containing leucine rich repeat sequences and more preferably the matrix is characterised by the fact that at least some of the collagen monomers have retained N-propeptide containing a polypeptide from a proteoglycan core protein.

The inventors have found that collagen matrices according to the fifth aspect of the invention have several advantages over known collagen matrices.

A first advantage of the collagen matrices according to the present invention is that the inventors have found, to their surprise, that the collagen matrices do not suffer from gel contraction to the extent that occurs in known collagens. This has the great advantage that the matrices may be used for cosmetic purposes, wound healing purposes and other industrial uses that until know have been blighted by the phenomenon of gel contraction or shrinkage.

A second advantage is that the proteoglycan group on the collagen matrix is able to bind growth factors.

An important function of the ECM is the storage and presentation of growth factors to cells. Proteoglycan components of the ECM play a central role in the regulation of the activity of a number of growth factors and therefore represent powerful pathophysiological modulators. The inventors have found that procollagens according to the third aspect of the invention may be incorporated into collagen matrices according to the fifth aspect of the invention and thereby enable artificial collagen matrices to bind growth factors.

Collagen matrices according to the fifth aspect of the invention are preferably made from human recombinant DNA molecules according to the second aspect of the invention. When this is the case, a third advantage is that the matrices are less likely to cause allergic and inflammatory responses when administered to humans.

Therefore, collagen polymers according to the fourth aspect of the invention may be used to form a macroscopic collagen matrix or gel according to the fifth aspect of the invention which has the added functional property of being able to bind growth factors and which, surprisingly is also resistant to gel contraction which may be caused by colonisation of the matrix with cells such as fibroblasts or epithelial cells.

Preferably, up to 50% of the collagen matrix comprises modified collagen according to the invention to provide the desired anti-shrinkage properties, more preferably 10-40%, especially 20-30%.

A collagen matrix may be formed by neutralising and warming acidic solutions of collagen monomers or procollagens (in the presence of suitable proteinases). Under such conditions the collagen monomers spontaneously self-assemble into polymeric fibrils that then become entangled to form a hydrated and porous gel. The rigidity of such a gel is, at least in part, dependent on the concentration of the collagen used to form the gel and on the diameter of the collagen fibrils formed. The collagen matrix or gel assumes the shape of the container in which it is formed. Therefore, gels can be made that are thin (millimetres) in one dimension and extensive (centimetres or larger) in other dimensions. Such matrices can be suitably shaped to form the basis of replacement skin or cornea. Alternatively, collagen gels can be cast in moulds that have the shape of long bones (cylindrical and long), jaw bones (sickle shaped or curved), articular cartilage (disc shaped), tendon (rope shaped) or ligament (shaped like a strap).

An example of a preferred matrix according to the fifth aspect of the invention is described in more detail in Example 10.

The fact that the matrices according to the fifth aspect of the invention do not shrink and are able to bind growth factors makes them useful for a number of applications, including:

1. Bodily implants. For instance, "stuffers" or packing agents for use surgically (especially for cosmetic surgery);
2. As modulators of collagen fibril formation;
3. As modulators of growth factor function;
4. Cell delivery systems (e.g. for drugs and pharmaceuticals);
5. As anti-cell adhesion systems;
6. As coatings for catheters and other medical products to increase cell adhesion or to have poor cell and bacterial adhesive properties;
7. For the synthesis of novel matrices for use in biology, medicine, science and industry; and

8. Novel medicaments.

Collagen polymers and matrices according to the fourth and fifth aspects of the invention may comprise exclusively recombinant collagen derived from modified procollagen molecules according to the invention. Alternatively such collagen polymers or matrices may be mixtures of modified collagens or modified procollagens according to the invention and collagen extracted from tissue or cell cultures, such as is available from commercial sources. For example, collagen polymers according to the fourth aspect of the invention may be combined with bovine type I collagen to form a matrix according to a fifth aspect of the invention.

Procollagens or collagens according to the present invention may be used to coat the surfaces of collagen fibrils in a gel or matrix formed from natural collagens (e.g bovine collagens) or they may be incorporated into the fibrils during gel formation. Proteoglycan moieties are thereby presented to the surface of the collagen fibrils where they can interact with cells or influence cellular function. The procollagens may be applied as a soluble precursor with a procollagen C-proteinase such as BMP-1 which converts the soluble procollagen to fibril-forming collagen having its N-terminal domain retained to allow gel formation *in situ.* This enables the modified collagen to integrate and mesh with collagen fibrils at the point of application.

Molecules according to the first – fifth aspects of the invention may be employed in a research setting for exploring a wide range of biological phenomenon from cell adhesion to wound healing and from cell differentiation and apoptosis to the manufacture of wound dressings with improved molecule and cell binding properties. However, a preferred use of the molecules is in the formation of collagen matrices which may be used for medical or cosmetic purposes.

According to a sixth aspect of the present invention there is provided the use of a molecule or matrix according to any one of the first - fifth aspects of the invention for the treatment of medical conditions.

According to a seventh aspect of the present invention there is provided the use of a molecule or matrix according to any one of the first - fifth aspects of the invention for the manufacture of a medicament for use in the treatment of wounds or fibrotic disorders.

According to a eighth aspect of the present invention there is provided a method of treating wounds comprising administering to a subject in need of treatment a therapeutically effective amount of a molecule or matrix according to any one of the first – fifth aspects of the invention.

It is preferred that the medical conditions treated are conditions that are at least partially characterised by remodelling of the ECM.

Molecules according the third and fourth aspects and matrices/gels according to the fifth aspect of the invention are particularly useful in the treatment of medical conditions because they can act as agents for delivering growth factors to a target tissue. The proteoglycan moiety of the N-propeptide on the procollagen or collagen (whether as a monomer or polymerised) is able to bind to a growth factor (e.g. TGF-β1). For example, this growth factor may be associated with the molecule according to the third or fourth aspects of the invention of the invention before the molecule is administered to a subject in need of treatment. The molecule will then circulate in the body and release the growth factor at a desired site. Alternatively the molecule may be administered without growth factor attached. When this is the case, the proteoglycan moieties will sequester growth factor *in vivo* and may act as a "pool" of growth factor at the target site.

Release of the growth factor may be dictated by its binding affinity with the proteoglycan moiety. Alternatively release of the growth factor may be regulated specifically (e.g. by designing the N terminal portion such that it sensitive to an agent (e.g. a proteolytic enzyme) found at the target site. Under these circumstances the modified procollagen or collagen will circulate with its growth factor "payload" and then release growth factor (because of cleavage from the N terminal end) at a target site.

It is to be appreciated that the molecule that may be pre-loaded onto the modified procollagen or collagen will depend upon the particular proteoglycan moiety incorporated into the procollagen or collagen.

Molecules according to the first – fourth aspects of the invention and matrices according to the fifth aspect of the invention are particularly useful for modulating the wound healing process.

Wound healing in adults is a complicated reparative process. The healing process begins with the recruitment of a variety of specialised cells to the site of the wound and involves ECM and basement membrane deposition, angiogenesis, selective protease activity and re-epithelialisation. An important component of the healing process in adult mammals is the stimulation of fibroblasts to generate the ECM which develops to repair the wound area. A further aspect of tissue healing and regeneration is the cell proliferative and cell inductive properties of cytokines. Relevant molecules include TGFβ and platelet derived growth factor, which have important functions in chemotaxis, cell proliferation and cell-cell signalling.

The connective tissue that forms during the healing process is often fibrous in nature and commonly forms into a connective tissue scar (a process known as fibrosis). A scar is an abnormal morphological structure resulting from a previous injury or wound (e.g. an incision, excision or trauma). Scars are composed of a connective tissue which is predominately a matrix of collagen types I and III and fibronectin. The scar may consist of collagen fibres in an abnormal organisation (as seen in scars of the skin) or it may be an abnormal accumulation of connective tissue (as seen in scars of the central nervous system). Most scars consist of abnormally organised or ectopically deposited collagen.

It is often desirable to increase the rate of healing in the case of acute wounds (such as penetrative injuries, burns, nerve damage or even wounds resulting from elective surgery), chronic wounds (such as diabetic, venous and decubitus ulceration) or for generally healing compromised individuals (for example the elderly). In these

examples, the wounds can severely influence quality of life or even result in death and therefore the rate of healing often needs to be increased as much as is clinically possible. Where the rate of wound healing is increased, there is often an associated increase in scar formation but this may be of secondary importance compared to the desired increase in the rate of healing.

The term "wound" as used herein is exemplified by, but not limited to, injuries to the skin. Other types of wound can involve damage, injury or trauma to an internal tissue or organ such as the central nervous system, lung, kidney, heart, gut, tendons, liver, and hollow organs including blood vessels and gut.

There are however other instances where the regulation of scar formation is of primary importance and the rate of wound healing is only of secondary consideration. Examples of such situations are scars of the skin where excessive scarring may be detrimental to tissue function and particularly when scar contracture occurs (for instance skin burns and wounds which impair flexibility of a joint). The reduction of scarring to the skin when cosmetic considerations are important is also highly desirable. Furthermore in the skin, hypertrophic or keloid scars (particularly in Afro-Caribbean and Mongoloid races) can cause functional and cosmetic impairment and there is a need to prevent their occurrence. Scarring resulting from skin grafts in both donor sites and from the application of artificial skin can also be problematic and need to be minimised or prevented.

As well as scars of the skin, internal scarring or fibrosis can be highly detrimental and specific examples include:

(i) Within the central nervous system, glial scarring can prevent neuronal reconnection (e.g. following neuro-surgery or penetrating injuries of the brain).

(ii) Scarring in the eye can be detrimental. In the cornea, scarring can result in abnormal opacity and lead to problems with vision or even blindness. In the retina, scarring can cause buckling or retinal detachment and consequently blindness. Scarring following wound healing in operations to relieve pressure in glaucoma (e.g. glaucoma filtration surgery) results in the failure of the surgery whereby the aqueous humour fails to drain and hence the glaucoma returns.

(iii) Scarring in the heart (e.g. following surgery or myocardial infarction) can give rise to abnormal cardiac function.

(iv) Operations involving the abdomen or pelvis, often result in adhesion between viscera. For instance, adhesions between elements of the gut and the body wall may form and cause twisting in the bowel loop leading to ischaemia, gangrene and the necessity for emergency treatment (untreated they may even be fatal). Likewise, trauma or incisions to the guts can lead to scarring and scar contracture to strictures which cause occlusion of the lumen of the guts which again can be life threatening.

(v) Scarring in the pelvis in the region of the fallopian tubes can lead to infertility.

(vi) Scarring following injury to muscles can result in abnormal contraction and hence poor muscular function.

(vii) Scarring or fibrosis following injury to tendons and ligaments can result in serious loss of function.

Related to the above is the fact that there are a number of medical conditions known as fibrotic disorders in which excessive fibrosis leads to pathological derangement and malfunctioning of tissue. Fibrotic disorders are characterised by the accumulation of fibrous tissue (predominately collagens) in an abnormal fashion within the tissue. Accumulation of such fibrous tissues may result from a variety of disease processes. These diseases do not necessarily have to be caused by surgery, traumatic injury or wounding. Fibrotic disorders are usually chronic. Examples of fibrotic disorders include cirrhosis of the liver, liver fibrosis, glomerulonephritis, pulmonary fibrosis, scleroderma, myocardial fibrosis, fibrosis following myocardial infarction, central nervous system fibrosis following a stroke or neuro-degenerative disorders (e.g. Alzheimer's Disease), proliferative vitreoretinopathy (PVR) and arthritis. There is therefore also a need for medicaments which may be used for the treatment of such conditions by regulating (i.e. preventing, inhibiting or reversing) fibrosis / scarring in these fibrotic disorders.

Whilst the above considerations mainly apply to conditions, disorders or diseases of man it will be appreciated that wound healing, scarring and fibrotic disorders can also be problematic in other animals, particularly veterinary or domestic

animals (e.g. horses, cattle, dogs, cats etc). For instance abdominal wounds or adhesions are a major reason for having to put down horses (particularly race horses), as are tendon and ligament damage leading to scarring or fibrosis.

In accordance with the sixth, seventh and eighth aspects of the invention, the inventors have established that collagen polymers or gels with N-propeptide domains modified according to the invention may be used for the treatment of wounds and fibrotic disorders. Depending on how the molecules or gels are used, various regulating effects may be provided for the treatment of wounds or fibrotic disorders and these are discussed more fully below.

The inventors have found that they can modulate wound healing in subtle ways. When molecules according to the third or fourth aspects of the invention are administered to a wound or fibrotic site they bind growth factors, such as TGF-β. Depending upon the nature of interaction between the proteoglycan or derivative thereof and the growth factor there can be at least two pathophysiological effects. TGF-β activity may be reduced by binding to a molecule according to the present invention. This results in reduced scarring and fibrosis but may possibly slow the rate of healing. Alternatively the TGF-β may remain active and/or the collagen polymer may act as a reservoir prolonging the half life of TGF-β at the wound site or site of fibrosis. This has the result of increasing the rate of healing but may be at the expense of scar formation. It will be appreciated that the molecular design of the N propeptide forming domain will dictate which of these effects will prevail.

A further medical use according to the sixth, seventh and eighth aspects of the invention is the addition of growth factor to a molecule or matrix according to the third, fourth or fifth aspects of the invention before it is administered to the subject. Under these circumstance the molecule or matrix may be "loaded" with TGF-β which is to be delivered at the desired site. Selective delivery can be achieved by making cleavage of the N-propeptides sensitive to an agent only found at the wound or fibrotic site. Alternatively selectivity may be conferred by introducing a second type of N-propeptide modified procollagen or collagen which binds to a particular cell type (e.g. the propeptide comprises a polypeptide receptor agonist that binds to a cell

specific receptor). Matrices loaded with growth factors are particularly useful for promoting the rate of wound healing.

It will be appreciated that the nature of the effect of molecules according to the invention on the wound healing process will depend upon what growth factor interacts with the molecule at the wound site or, alternatively what growth factor is "loaded" onto the molecule. For instance administration of a molecule preloaded with TGF-β1 causes a wound to heal quickly but may result in a scar whereas administration of a molecule preloaded with TGF-β3 causes a wound to heal without causing excessive scarring.

Molecules according to the third and fourth aspects of the invention and a matrix according to the fifth aspect of the invention may be formulated into a various types of medicament. The medicament of the invention may take a number of different forms depending, in particular on the manner in which the medicament is to be used. Thus, for example, the medicament may be in the form of a liquid, ointment, cream, gel, hydrogel, powder, aerosol or an implantable device (e.g. by conjugation to a biopolymer sponge).

Molecules according to the third and fourth aspects of the invention may be administered directly (e.g. in liquid form). However, it is preferred that the molecules are incorporated into a wound dressing, an implantable device, artificial skin or tissue etc.

It is preferred that the medicaments are for topical application. The medicament may be most suitably used for topical application to the skin or wound area.

Medicaments comprising modified procollagens, collagens or collagen fibrils may be delivered by means of an aerosol (e.g. for delivery to fibrotic conditions of the lung).

It will be appreciated that the vehicle of the medicament should be one which is well tolerated by the patient and allows release of the collagen polymer, and/or release

of any conjugated growth factor, to the wound or site of fibrosis. The vehicle will ideally be sterile and may be combined with excipients and / or stabilizers as well as the molecule to form the medicament. Such a vehicle is preferably biodegradeable, bioresolvable, bioresorbable and/or non-inflammatory.

The medicament may be used in a number of ways. Thus, for example, it may be applied in, and/or around a wound of a patient to provide the desired promotion of wound healing. If the composition is to be applied to an "existing" wound, then the pharmaceutically acceptable vehicle will be "mild" enough such that it does not cause an inflammatory response or is toxic to the tissue.

Molecules according to the third or fourth aspects of the invention may be provided on a sterile dressing or patch which may be used to cover or even pack a wound or fibrotic site.

The medicament may be provided as an implantable device from which it may be released better. For instance, it may be released by biological dissolution or degradation of the device. Alternatively an external stimulus, such as ultrasound, may cause release of the procollagen, collagen monomer or collagen polymer.

It is also possible to use medicaments in accordance with the invention in a prophylactic manner. For instance, the medicament may be applied prior to surgery so as to provide for regulation of healing of the subsequently formed surgical wound.

The collagen matrices according to the fifth aspect of the invention are also useful for antifibrotic or antiscarring applications. In this case the matrices may be loaded with a non-fibrotic growth factor (e.g. TGF-β3).

A collagen matrix may then be administered to a subject (e.g. to the skin, cartilage, muscle or neural tissues) in the form of a semi-solid gel. Alternatively a more solid matrix may be formed which may be used in the formation of a wound dressing, an implantable device, artificial skin or tissue etc.

A major problem with conventional collagens is that the collagen shrinks during the healing process. The inventors believe this is caused by epidermal cells or fibroblasts attaching to the matrix. The cells grow and proliferate and thereby cause the collagen to contract. This can result in unhealed areas of a wound becoming exposed. Matrices according to the fifth aspect of the invention do not suffer from shrinkage (or at least suffer to a lesser extent then known collagen gels). Although we do not wish to be bound by any hypothesis, we believe this may be because the proteoglycan moieties provide anchorage points for cell attachment and thereby obviate the need for the cells to bind directly to collagen monomers and cause shrinkage.

Artificial skins comprising matrices according to the fifth aspect of the invention may comprise ECM components alone or may further comprise cultured cells such as fibroblasts and/or endothelial cells. Artificial skins containing such cells are known as "living" replacement skin products. Both types of artificial skin are particularly useful because they may be laid over the wound or burn and do not suffer from the shrinkage associated with known collagens.

It is preferred that the collagen matrices are formed into artificial skin for topical application to dermal wounds or burns. The artificial skins comprising matrices according to the fifth aspect of the invention are particularly useful for treating severe wounds, extensive wounds, chronic wounds (e.g. dermal ulcers) and burns.

It will be appreciated that the matrix should be hydrated in a pharmaceutically acceptable vehicle. The vehicle should be sterile and "mild" enough such that it does not cause an inflammatory response or is toxic to the tissue being treated.

The matrix may be incorporated into a sterile dressing or patch which may be used to cover or even pack a wound or fibrotic site.

In a preferred embodiment, the matrix is applied to a dressing, such as a Combiderm N dressing and then dehydrated. The dehydrated gel carried on the dressing is then applied to a wound.

The matrix may be provided as an implantable device from which the matrix *per se* may be released into the wound site. Alternatively the collagen may be retained in the device and growth factor bound to the proteoglycan moieties may be released therefrom. Release may be caused by biological dissolution or degradation of the device. Alternatively an external stimulus, such as ultrasound, may cause release of the collagen polymer and/or the growth factor.

A collagen matrix according to the fifth aspect of the invention may be cast into a sheet. Preferred sheets may be 1- several millimetres thick by several centimetres square. Such sheets can be acellular or populated with mesenchymal and/or fibroblastic cells to generate an artificial skin, cartilage, bone or cornea, or endothelial cells to produce cardiovascular patches. The cells may be obtained from a patient or a tissue-matched donor, stem cells from a patient or a donor, or cells that have been amplified in culture. Such matrices may be coated with molecules according to the third and fourth aspects of the invention to confer extra-growth factor binding functionality to the matrix. The collagen matrix or collagen-cell construct can be stored under aseptic conditions and at physiological temperatures or under cryogenic storage conditions until needed.

It will be appreciated that the amount of molecule required to modulate healing and fibrosis depends on a number of factors such as its biological activity and bioavailability, which in turn depends on the mode of administration and the physicochemical properties of the particular molecule used. For example, the amount of collagen matrix required will depend upon factors such as the concentration of the gel (this may be required to be aqueous, viscous or relatively solid – depending upon the clinical need), the proportion of collagens with proteoglycan moieties contained therein and the amount of growth factor bound thereto (if any). Other factors include:

A) The specific condition to be treated.

B) The severity of the condition.

C) The age of the subject.

D) The site of delivery.

E) The half-life of the molecule in the subject being treated.

The frequency of administration will also be influenced by the above mentioned factors and particularly the half-life of the compound, matrix or its growth factor payload within the subject being treated.

Generally, a subject being treated will derive benefit from the application of the modified procollagen, collagen monomer or collagen polymer, if it as administered to a wound within 7 days of wounding, preferably within 48 hours of wounding, more preferably within 24 hours of wounding and even more preferably within 12 hours of wounding. The medicament should be administered to a subject suffering from a fibrotic condition according to a clinicians directions. This may be as soon as diagnosis has occurred. Therapy should continue until the wound has healed or fibrotic disorder cleared to a clinicians satisfaction.

When used as a prophylactic (e.g. before surgery) the medicament should be administered as soon as it is recognised that a wound may occur or fibrotic disorder may develop. For instance, a cream or ointment containing collagen polymer loaded with a growth factor may be applied to a site on the skin of a subject where elective surgery is to be performed and an increased rate of wound healing is subsequently desired. In this case, the medicament may be applied during the preoperative preparation of the subject or it may even be desirable to apply it in the hours or days preceding the surgery (depending upon the health status and age of subject as well as the size of the wound to be formed).

Frequency of administration will depend upon the biological half-life of the molecule used. Typically a cream or ointment should be administered to a target tissue such that the concentration of the molecule at the wound site is maintained at a level suitable for having a therapeutic effect. This may require administration daily or even several times daily.

Known procedures, such as those conventionally employed by the pharmaceutical industry (e.g. *in vivo* experimentation, clinical trials etc), may be used to establish specific formulations of compositions and precise therapeutic regimes (such as daily doses of the compounds and the frequency of administration).

Generally, for use in accordance with the invention a medicament containing an amount of 1ng to 10mg of collagen polymer, more preferably 1μg to 1mg of collagen polymer, may be applied per centimetre of linear wound. Purely by way of example, a medicament containing about 10μg collagen polymer is suitable for application to a 1 cm linear incisonal wound. Higher doses are required to stimulate the healing of chronic wounds compared to acute wounds.

Efficacy of medicaments, and particularly those formulated for application to chronic wounds, have enhanced efficacy when combined with a protease inhibitor (e.g. galadrin) Protease inhibitors prevent or retard the degradation of the collagen by proteases which may be found in high levels in wounds, particularly chronic wounds. The protease inhibitor is preferably a broad spectrum protease inhibitor.

It will be appreciated that the molecules and matrices according to the third, fourth and fifth aspects of the invention may be used in combination with other wound healing or anti-fibrotic agents or followed by another agent (e.g. for prevention of scarring).

Another preferred use of collagen matrices according to the fifth aspect of the invention is as a stuffer in cosmetic surgery. Accordingly, a ninth aspect of the invention provides the use of a matrix according to the fifth aspect of the invention for use in cosmetic treatment. For instance, the matrices may be used as an advanced filler material in facial asthetic procedures. The anti-shrinkage properties of the matrix result in this filler material being particularly suitable for cosmetic applications, such as collagen injections.

It will be appreciated that matrices according to the fifth aspect of the invention (used to treat medical conditions, cosmetically or otherwise) may be formed *in situ* (i.e. at the tissue/site where the matrix is required). For instance, a solution or slurry of collagen polymers according to the fourth aspect of the invention may be used to soak a wound dressing. Gel formation may be induced when the dressing is used (e.g. a reaction may initiated when the dressing is removed from its package or contacts a wound site). Alternatively a solution of collagen polymers according to the fourth

aspect of the invention, or even procollagens according to the third aspect of the invention may be injected into a target body tissue and matrix formation allowed to proceed with native collagens.

DNA molecules according to the second aspect of the invention may be used in gene therapy techniques. Therefore according to a tenth aspect of the present invention there is provided a delivery system for use in a gene therapy technique, said delivery system comprising a DNA molecule according to the second aspect of the invention which is capable of being transcribed to lead to the expression of a modified pro-α chain according to the first aspect of the invention at a wound site or site of fibrosis.

According to an eleventh aspect of the present invention there is provided the use of a delivery system as defined in the preceding paragraph for use in the manufacture of a medicament for treating wounds or fibrotic disorders.

According to a twelfth aspect of the present invention there is provided a method of treating a wound or fibrotic condition which consists of administering to a patient in need of treatment a therapeutic dose of a delivery system as defined above.

The delivery systems are highly suitable for achieving sustained levels of a procollagen molecule according to the third aspect of the invention or a collagen polymer according to the fourth aspect of the invention at a wound site or site of fibrosis over a longer period of time than is possible for most conventional delivery systems. Modified pro-α chains may be continuously expressed from cells at the site that have been transformed with the DNA molecule of the second aspect of the invention. Therefore, even if the modified procollagen or collagen polymer has a very short half-life as an agent *in vivo*, therapeutic doses may be continuously expressed from the treated tissue.

Furthermore, the delivery system of the invention may be used to provide the DNA molecule without the need to use conventional pharmaceutical vehicles such as those required in ointments or creams that are contacted with the wound or site of

fibrosis. This is particularly beneficial as it can often be difficult to provide a satisfactory vehicle for a compound for use in wound healing (which are required to be non-inflammatory, biocompatible, bioresorbable and must not degrade or inactivate the active agent (in storage or in use)).

The delivery system is such that the DNA molecule is capable of being expressed (when the delivery system is administered to a patient) to produce modified pro-α chains which form procollagens and then collagen polymers with proteoglycan modified N terminals. These modified N terminals then interact with growth factors at the site of the wound or fibrosis and thereby treat the condition.

The DNA molecule may be contained within a suitable vector to form a recombinant vector. The vector may for example be a plasmid, cosmid or phage. Such recombinant vectors are highly useful in the delivery systems of the invention for transforming cells with the DNA molecule. The vector may be pCEP4 or a similar vector.

Recombinant vectors may also include other functional elements. For instance, recombinant vectors can be designed such that the vector will autonomously replicate in the nucleus of the cell. In this case, elements which induce DNA replication may be required in the recombinant vector. Alternatively the recombinant vector may be designed such that the vector and recombinant DNA molecule integrates into the genome of a cell. In this case DNA sequences which favour targeted integration (e.g. by homologous recombination) are desirable. Recombinant vectors may also have DNA coding for genes that may be used as selectable markers in the cloning process.

The recombinant vector may also further comprise a promoter or regulator to control expression of the gene as required.

The DNA molecule may (but not necessarily) be one which becomes incorporated in the DNA of cells of the subject being treated. Undifferentiated cells may be stably transformed leading to the production of genetically modified daughter cells (in which case regulation of expression in the subject may be required e.g. with

specific transcription factors or gene activators). Alternatively, the delivery system may be designed to favour unstable or transient transformation of differentiated cells in the subject being treated. When this is the case, regulation of expression may be less important because expression of the DNA molecule will stop when the transformed cells die or stop expressing the protein (ideally when the wound, fibrosis or scarring has been treated or prevented).

The delivery system may provide the DNA molecule to the subject without it being incorporated in a vector. For instance, the DNA molecule may be incorporated within a liposome or virus particle. Alternatively the "naked" DNA molecule may be inserted into a subject's cells by a suitable means e.g. direct endocytotic uptake.

The DNA molecule may be transferred to the cells of a subject to be treated by transfection, infection, microinjection, cell fusion, protoplast fusion or ballistic bombardment. For example, transfer may be by ballistic transfection with coated gold particles, liposomes containing the DNA molecule, viral vectors (e.g. adenovirus) and means of providing direct DNA uptake (e.g. endocytosis) by application of plasmid DNA directly to the wounded area topically or by injection.

Whilst the above considerations mainly apply to wounds of man it will be appreciated that wound healing, can also be problematic in other animals (especially veterinary and domestic animals such as cattle, horses, dogs, cats etc). For instance, abdominal wounds or adhesions are a major reason for having to put down horses. The medicaments and delivery systems discussed above are also suitable for use in the healing of such animals.

The present invention will now be further described with reference to the following non-limiting examples and figures in which:

Figure 1 schematically illustrates a natural procollagen molecule;

Figure 2 schematically illustrates dec-procollagen, a procollagen molecule according to the third aspect of the invention;

Figure 3 illustrates the nucleotide sequence of a DNA molecule according to the second aspect of the invention from Example 1;

Figure 4 illustrates the amino acid sequence of a modified pro-α chain according to the first aspect of the invention from Example 1;

Figure 5 is a photograph of a Western blot referred to in Example 3;

Figure 6 is a photograph of a silver stained gel referred to in Example 4;

Figure 7 is a plot showing quantitation of platelet derived growth factor binding to a molecule according to the third aspect of the invention referred to in Example 5;

Figure 8 is a plot showing quantitation of transforming growth factor beta binding to a molecule according to the third aspect of the invention referred to in Example 5;

Figure 9 is a plot showing quantitation of human fibroblasts binding to a molecule according to the third aspect of the invention referred to in Example 6 wherein (A) represents binding of human skin fibroblasts to a molecule according to the third aspect of the invention, (B) represents binding of human fibroblasts to fibronectin, and (C) is a comparison of binding of human skin fibroblasts to a molecule according to the third aspect of the invention and fibronectin in the presence of 1 mM MnCl;

Figure 10 is a plot showing quantitation of COS-7 cells binding to a molecule according to the third aspect of the invention referred to in Example 6 wherein (A) represents binding of COS-7 cells to a molecule according to the third aspect of the invention in the presence and absence of divalent cations, and (B) represents binding of COS-7 cells to human fibronectin in the presence and absence of divalent cations;

Figure 11 is a block diagram illustrating the detection of human decorin in dec-proα1 (III) and pNdec™ protein gel samples. Data represents the mean +/- standard deviation of 4;

Figures 12 and 13 are microscopic cross-sectional images (x 5 and x 20 respectively) of an unmanipulated wound (a) and a wound treated with a modified procollagen gel pNdec™ according to one aspect of the invention (b);

Figures 14, 15 and 16 respectively show a block diagram illustrating the wound area, percentage epithelialization and cell numbers/mm^2 6 days post wounding for unmanipulated wounds and wounds treated with a gel of the present invention;

Figure 17 is a photograph showing that a molecule according to the third aspect of the invention attenuates contraction of type I collagen gels by human skin fibroblasts for up to 6 days in culture as referred to in Example 10;

Figures 18a to 18c are photographs showing the shrinkage of an unmodified collagen gel between 2, 4 and 70 hours respectively;

Figures 19a to 19c are photographs showing the reduced shrinkage exhibited by a pNdec™ gel according to the invention between at 2, 4 and 70 hours respectively;

Figure 20 is a graph illustrating the amount of collagen gel contraction in the presence and absence of pNdec™ over 150 hours;

Figures 21 and 22 are respectively a top view and side view of a dressing having a wet collagen gel according to the invention applied thereto; and

Figures 23 and 24 are respectively a top view and side perspective view of the dressing shown in Figures 21 and 22 with the collagen gel dried thereon.

Figure 1 illustrates a natural procollagen with an N-terminal propeptide 1, alpha helical domain 2 and a C-terminal propeptide 3. A procollagen N-Proteinase cleavage site 4 in the hinge region of the molecule (between 1 and 2) is also illustrated. Figure 2 illustrates dec-proα1(III) or Prodec™ a procollagen molecule according to the third aspect of the invention in which the N propeptide 1 has been replaced by decorin 5.

EXAMPLE 1: Design and Construction of a DNA molecule according to the second aspect of the invention.

A DNA molecule according to the second aspect of the invention was constructed comprising the entire coding region for decorin in place of the globular domain of the N-propeptide of the proα1(III) chain.

First a PCR product was prepared containing the entire coding region for decorin but lacking its natural stop codon. To facilitate subsequent cloning and fusion with the type III procollagen sequence a Not I restriction enzyme site was introduced

within the sense PCR primer and the overlapping sequence for type III procollagen within the antisense PCR primer.

The template for the PCR reaction was a decorin cDNA clone (NCBI XM_012239) with the following sequence:

ggt tccctggttg tgaaaataca tgagataaat catgaaggcc actatcatcc
tccttctgct tgcacaagtt tcctgggctg gaccgtttca acagagaggc ttatttgact
ttatgctaga agatgaggct tctgggatag gcccagaagt tcctgatgac cgcgacttcg
agccctccct aggcccagtg tgccccttcc gctgtcaatg ccatcttcga gtggtccagt
gttctgattt gggtctggac aaagtgccaa aggatcttcc ccctgacaca actctgctag
acctgcaaaa caacaaaata accgaaatca aagatggaga ctttaagaac ctgaagaacc
ttcacgcatt gattcttgtc aacaataaaa ttagcaaagt tagtcctgga gcatttacac
ctttggtgaa gttggaacga ctttatctgt ccaagaatca gctgaaggaa ttgccagaaa
aaatgcccaa aactcttcag gagctgcgtg cccatgagaa tgagatcacc aaagtgcgaa
aagttacttt caatggactg aaccagatga ttgtcataga actgggcacc aatccgctga
agagctcagg aattgaaaat ggggctttcc agggaatgaa gaagctctcc tacatccgca
ttgctgatac caatatcacc agcattcctc aaggtcttcc tccttccctt acggaattac
atcttgatgg caacaaaatc agcagagttg atgcagctag cctgaaagga ctgaataatt
tggctaagtt gggattgagt ttcaacagca tctctgctgt tgacaatggc tctctggcca
acacgcctca tctgagggag cttcacttgg acaacaacaa gcttaccaga gtacctggtg
ggctggcaga gcataagtac atccaggttg tctaccttca taacaacaat atctctgtag
ttggatcaag tgacttctgc ccacctggac acaacaccaa aaaggcttct tattcgggtg
tgagtctttt cagcaacccg gtccagtact gggagataca gccatccacc ttcagatgtg
tctacgtgcg ctctgccatt caactcggaa actataag

(SEQ ID No. 1)

The sequence of the PCR oligonucleotides was as follows: (all 5'-3')

5' end (including NotI site):

CACGTTACTGAGCGGCCGCACTGTTCCCTGGTTGTGAAAATAC

(SEQ ID No. 2)

3' end:

GCCTTGAGGTCCTTGACCATTCTTATAGTTTCCGAGTTGAATGG

(SEQ ID No. 3)

The sequence overlapping the proα1(III) sequence at the junction site is shown underlined on PCR oligonucleotide of Seq ID No. 3.

The inventors also prepared a PCR product that contained a sequence from the proα1(III) chain up to the junction site (i.e. at the end of the N-propeptide globular domain) to a unique BamHI restriction enzyme site within the triple helical domain. The template for this reaction was a proα1(III) chain cDNA (The sequence of this template is publicly available as NCBI X14420)

The PCR oligonucleotides used with the proα1(III) chain template were as follows:

5' end:

AATGGTCAAGGACCTCAAGGC

(SEQ ID NO. 4)

SEQ ID No 4 is complementary to the overlap region of oligonucleotide 2 (SEQ ID NO. 3) above (the underlined region).

3' end:

AGACCCTGCAGGTCCAACTT

(SEQ ID NO. 5)

The two PCR products (from primers of SEQ ID Nos 2 & 3 and 4 & 5) were then purified and mixed and a secondary PCR reaction carried out to join the two PCR products together and to amplify the resulting joined product.

The 5' and 3' oligonucleotides used in the secondary PCR reaction were:

5' end:

CACGTTACTGAGCGGCCGCACTGTTCCCTGGTTGTGAAAATAC

(SEQ ID NO. 6)

6. 3' end

AGACCCTGCAGGTCCAACTT

(SEQ ID NO. 7)

The PCR product resulting from the secondary PCR reaction was then digested with Not I and BamHI and subcloned into the BamHI site within the coding region of the proα1(III) cDNA (NCBI X14420).

The resulting construct is a DNA molecule according to the second aspect of the invention and contains the entire coding region for decorin (including the amino terminal signal sequence) that replaces the globular domain from the N-propeptide of the proα1(III) chain. The entire nucleotide sequence of the DNA molecule is presented in Fig 3 (and SEQ ID No. 8). Fig.4 (and SEQ ID No. 9) represents the amino acid sequence of the modified pro-α chain (a molecule according to the first aspect of the invention) which may be expressed from the DNA molecule. The junction between the decorin and procollagen sequences is shown as underlined in Figs 3 and 4.

EXAMPLE 2: Expression of modified procollagens according to the third aspect of the invention.

The DNA molecule described in Example 1 was sub-cloned into the expression vector PCEP4 (Invitrogen Life Technologies) for expression in HEK293-EBNA cells (Invitrogen Life Technologies).

The PCEP4 vector is commercially available and the sequence may be found at http://www.invitrogen.com.

HEK293-EBNA cells are known to those skilled in the art and details are available from http://www.invitrogen.com/Content/Tech-Online/molecular_biology/manuals_pps/293ebna_man.pdf

HEK293-EBNA cells do not secrete procollagens and so are ideal for a negative background to express collagens in. Importantly, these cells do contain prolyl 4-hydroxylase which is vital for the hydroxylation of proline residues in the procollagen sequence and hence for the stability of the triple helix. The HEK293-EBNA line also expresses the EBNA-1 antigen that ensures that any plasmid DNA transfected into the cell is maintained episomally when the presence of that plasmid is selected for by the appropriate antibiotic (generally hygromycin).

Modified pro-α chains according to the first aspect of the invention are generated in the endoplasmic reticulum of the HEK293-EBNA cells. These molecules then automatically form a homotrimer (modified procollagen molecules according to the third aspect of the invention). The modified procollagen molecule produced from said cells is hereinafter referred to interchangeably as dec-proα1(III) or Prodec™.

EXAMPLE 3: Characterisation of dec-proα1(III) (a modified procollagen according to the third aspect of the invention).

A Integra CL 350 flask was seeded with HEK293-EPNA cells transformed with the DNA molecule from Example 1 and left for 7 days. The enriched medium was then harvested three times weekly (days 7, 9, 12, 14 and 16 after seeding).

Dec-proα1(III) was initially characterised by Western blotting using 10 μL of cell harvest supernatant per lane on a 6% SDS-PAGE gel. The presence of dec-proα1(III) was detected using an antibody that specifically recognises the collagen helical domain (αcolIII at 1:500 dilution). The antibody was a rabbit anti-human type 1 antibody (available from AMS-BIOTECH Cat. No. TS9103R)

The results are presented in Figure 5 and show that the cells produce dec-proα1(III) secreted into the medium for up to 16 days of culture.

Digesting the supernatants with chymotrypsin/trypsin, (denoted as CT/T in Fig. 5) caused the removal of the C and N-propeptides and leaves the triple helical domain intact. Such treatment was therefore useful for assessing the presence of triple helical dec-proα1(III). The dec-proα1(III) produced from the cells was partially resistant to degradation demonstrating that triple helical molecules had indeed been produced.

EXAMPLE 4: Purification of dec-proα1(III) (a modified procollagen according to the third aspect of the invention).

Dec-proα1(III) or Prodec™ was purified from cell-culture medium after harvesting from the transfected cells. The procedure involved anion-exchange followed by size-exclusion chromatography.

4.1 Anion-exchange chromatography. Cell-culture medium was centrifuged at 3000 rpm for 5 minutes to remove cellular debris. This solution was then applied to a Perseptive Biosystems POROS HQ10 (10 x 100mm) anion-exchange column equilibrated with 50mM Tris pH 8.0, at a flow rate of 16 ml/min. Bound protein was eluted from the column with a 0–1.2M NaCl gradient over 20 column volumes. Dec-proα1(III) was eluted between 0.8 and 1.2M NaCl.

4.2 Size-exclusion chromatography. The dec-proα1(III) fraction from the anion-exchange step was applied to a Waters Protein-Pak SW300 (7.8 x 300mm) size-exclusion column equilibrated with 50mM Tris, 50mM EDTA, 300mM NaCl pH 8.0, at a flow rate of 0.5 ml/min. dec-proα1(III) eluted as the first major peak, at 7-8 ml.

4.3 Detection. Western blotting was used to identify fractions containing dec-proα1(III), at each stage of the purification.

4.4 Yield. Typically, 0.5mg of dec-proα1(III) was purified from 70ml of cell-culture medium.

A silver stain 10% SDS-PAGE gel of the fractions from each stage of purification is shown in Figure 6. Dec-proα1(III) appears as a smeared band at 220 kDa. In Figure 6 Lane 1 = molecular weight markers; Lane 2 = cell culture medium; Lane 3 = the pool of proteins eluted from the anion exchanger; and Lane 4 = protein eluted from the size exclusion chromatography column.

EXAMPLE 5: Growth factor and cytokine binding to dec-proα1(III)

Purified dec-proα1(III) (0-20 μg/ml) from Example 4 was immobilised to the surface of plastic ELISA plates (Immulon 1B, Dynex). Bovine serum albumin (BSA) was used in negative controls and as the blocking agent. The cytokines studied were recombinant human transforming growth factor beta (TGFβ) 1 and 3, platelet derived growth factor (PDGF) isoforms AA, AB and BB, and basic fibroblast growth factor (bFGF). The cytokines were obtained from R&D Systems. The catalogue numbers were 240-B (TGFβ1), 243-B3 (TGFβ3), 221-AA (PDGFAA), 222-AB (PDGFAB), 220-BB (PDGFBB) and (bFGF). The corresponding anti-cytokine antibodies used to detect cytokine binding were biotinylated and obtained from R&D Systems. The concentration of cytokines in the assays was 0.5 μg/ml.

5.1 PDGF binding.

The AB and BB isoforms of PDGF exhibited saturatable binding to dec-proα1(III) when 1-5 μg/ml of dec-proα1(III) was incubated with the ELISA plate surface. The AA isoform of PDGF demonstrated no binding for dec-proα1(III) at a concentration of 20μg/ml of dec-proα1(III). The data are presented in Figure 7 which illustrates a solid-phase assay of PDGF (rhPDGF at 0.5μg/ml) binding to dec-proα1(III).

5.2 TGFβ binding.

TGFβ1 exhibited saturatable binding to dec-procollagen at a concentration of 1 μg/ml of dec-proα1(III) incubated with the ELISA plate surface. TGFβ3 exhibited an apparent lower affinity for binding of dec-proα1(III). The results are shown in Figure 8.

5.3 bFGF binding.

bFGF showed no specific binding to dec-procollagen (data not shown).

EXAMPLE 6: Cell binding to dec-proα1(III).

Purified dec-proα1(III) (0-20 μg/ml) from Example 4 was immobilised to the surface of plastic tissue culture plates (Costar). Human placental fibronectin (from

Gibco) was used in positive controls of cell binding. Heat denatured bovine serum albumin (BSA) was in cation-free phosphate buffered saline (PBS) and was the blocking agent (10 mg/ml). The same was used in negative controls of cell binding. Cell binding as carried out at 37°C for 15 minutes. To investigate the role of integrins in cell binding, the assay was repeated in the presence of Mg, Mn and Ca cations. The cells were primary human foreskin fibroblasts (passage number 12 and 13) and COS-7, separately. The number of living cells in each measurement was 50,000.

The results for fibroblast binding to dec-proα1(III) are shown in Figure 9. The results for COS-7 cell binding to dec-proα1(III) are shown in Figure 10. The results show that dec-proα1(III) supports the binding of fibroblasts and COS-7 cells. Moreover, the binding was strongly dependent on cations, especially Mn^{2+} ions, indicative of integrin-mediated binding. In the presence of Mn^{2+} ions the binding of fibroblasts to dec-proα1(III) and to fibronectin were approximately equivalent.

EXAMPLE 7: Formulation of Medicaments for use in wound healing.

7.1 Dec-proα1(III) was incubated with an equimolar concentration of TGF-β1 to allow binding (see Example 5). The Dec-proα1(III)/ TGF-β1 was then diluted in sterile saline solution for injection down the margins of an incisional wound of the dermis.

7.2 Dec-proα1(III) was incubated with TGF-β3 to allow binding (see Example 5). The Dec-proα1(III)/ TGF-β3 was then combined with a sterile solution for use in an inhaler for administration as an aerosol.

EXAMPLE 8: Preparation of pNDec gels according to a fifth aspect of the invention and detection of the decorin moiety of Dec- proα1(III) and pNdec via ELISA methodology

Preparation of pNDec gels

Purified Dec-proα1 (III) or Prodec™ at known concentration, as estimated by BCA assay, was dialysed against bone morphogenetic protein (BMP-1-FLAG) cleavage buffer (1L) using a Slide-A-Lyzer cassette. Dialysis was performed at 4°C for 1 hr. An equal volume of water reconstituted BMP 1 FLAG was mixed with the dialysed Dec-proα1. The mixture was incubated in a water bath at 37°C overnight. This was known to be sufficient to effect removal of the C-propeptide of the Dec-proα1 (III) to generate pNdec™ *in situ*. "pNdec" comprises procollagens having their N-terminal domain retained but their C-terminal domain removed. The pNdec™, without further purification, was mixed appropriately with sterile: Rat tail Type I collagen, phosphate buffer, acetic acid and deionised water. Aliquots (1.5 ml) of the resulting mixture was placed in a non-stick sterile bacteriological dish (35 mm diameter). The dish was placed in a incubator at 37°C under 5%CO2 atmosphere for 3 hr to allow collagen gelation.

Table 1 below shows dilutions to make pNdec™ gels at the different concentrations.

Table 1

	50µg/ml	**150µg/ml**	**250µg/ml**	**360µg/ml**
Rat tail collagen @ 3000µg/ml	1.66 ml	1.66 ml	1.66 ml	1.66 ml
***pNdec @ 675 µg/ml**	0.37 ml	1.11 ml	1.85 ml	2.66 ml
Phosphate buffer I = 1.0	0.5 ml	0.5 ml	0.5 ml	0.5 ml
Acetic acid 10mM	0.18 ml	0.18 ml	0.18 ml	0.18 ml
Sterile deionised water	2.29 ml	1.56 ml	0.81 ml	0

* 50:50 (BMP1-Flag harvest + Dec-proα1(III) @ 1350 µg/ml) = 675 µg/ml

Detection pNdec in gels: an ELISA protocol

The gels were washed (precast gels from 200 µl solution) with 2ml PBS-T (Tween 20; 0.05%) and dissolved with freshly made 0.5 M acetic acid (1 ml) using a vortex

mix. 1ml of this solution was diluted to 5ml with $Na_2CO_3/NaHCO_3$ (50 mM, pH 9.6) and a 100 µl/well of the resultant solution was coated on Immulon 4HBX plate. The plate was left at 4°C overnight. The solution was decanted and the wells were washed (1x200 µl) with phosphate buffer saline containing 0.05% Tween 20;PBS-T. The wells were blocked with Superblock 200 µl/well and the plate immediately emptied by inversion (Repeat 3x).

100 µl of LF 136 (1:1000; rabbit human anti-decorin) was added. Antibody dilution was made up in a 10 fold dilute solution of Superblock in phosphate buffered saline and left at room temperature for 1hr. The solution was decanted and washed (3 x 200 µl) with PBS-T. 100 µl/well of 1:1000 donkey anti-rabbit HRP was added. This antibody was also diluted in a 10-fold dilution of Superblock. This was left at room temperature for 1hr. The solution was decanted and washed (3 x 200µl) with PBS-T.

100µl/well of QuantaBlu™ Working solution (9ml of QuantaBlu™ Sustrate solution + 1ml of QuantaBlu™ Stable peroxide substrate solution) was added and left at room temperature for 30 mins. 100µl/well of stop solution was added and the contents of replicates wells were pooled into cuvette (this step enabled sufficient volume of test sample (e.g. 12 wells with 200 µl/well resulted in sufficient volume to read in a cuvette (3 ml max. vol). Fluorescence measurements were recorded on a LS50B Perkin-Elmer fluorimeter set at excitation/slit width (325 nm/5 nm) and emission/slit width 420 nm/2.5nm, respectively. Integration period was set at 10 s with a 1% attenuation.

Collagen gel, dec-proα1(III) gel and pNdec™ gel were formed from a 200 µl gel solution. The gels were subsequently dissolved and diluted as previously stated in the above-mentioned ELISA protocol. The concentration of dec-proα1(III) and pNdec™ in the stock solution for the gel samples was 360 µg/ml (for 200 µl, dec-proα1(III) and pNdec™ content is 72 µg). This was further diluted with acetic acid and Na_2CO_3 as discussed in the ELISA protocol. Thus, for dec-proα1(III) and pNdec gel samples, the maximum amount coated for both proteins from the gel samples was 1.44 µg. Dec-proα1(III) and pNdec™ refer to neat protein (not in gel form) samples coated at 20 µg/ml (100 µl/well, max. protein coat is 2 µg).

Results

The data show a significant difference between all sample containing the human decorin moieties relative to the control with no human decorin (see Figure 11). This indicates a positive detection of the human decorin moiety in the appropriate samples. The bars labelled as Prodecol (Prodec™) and pNdec showed similar level of detection signal. These proteins were both coated at similar concentrations. Furthermore, the detection signal for dec-proα1(III) and pNdec™ were significantly higher than that of their counterparts in gel samples. The difference could be because the maximum surface coat of neat dec-proα1(III) and pNdec™ is 2 µg compared to a maximum surface coat of gel derived dec-proα1(III) and pNdec™ at 1.44 µg. The data also show a difference in detection signal between dec-proα1(III) gel and pNdec™ gel samples. This difference may be due to fibrillar incorporation of the pNdec. Thus, in the case of pNdec™, the novel protein is incorporated into fibrils and is refractory to being washed out of the gels.

Thus, the decorin moiety of dec-proα1(III) (procollagen (III)-decorin (human) and pNdec (the product generated when treated with BMP-1-FLAG) can be detected via ELISA methodology with LF 136 and a fluorogenic substrate.

EXAMPLE 9: Investigation into the use of a collagen matrix/gel according to a fifth aspect of the present invention in the healing of rat dermal excisional wounds.

8 week old male Sprague-Dawley rats were used for a dermal excisional wound study. Animals were anaesthetized with isofuorane/nitrous oxide/oxygen, the dorsum shaved and cleaned with alcohol, and four 6mm dorsal full-thickness excisions were made through the panniculus carnosus muscle using a punch biopsy. The wounds were treated with the following, in a rotational manner to avoid site bias:

1) 2 wounds unmanipulated
2) 3 wounds with collagen gel containing dec-proα 1 (III) or Prodec™ – a modified procollagen according to a third aspect of the present invention

Each gel had a layer of Combiderm™ dressing at the surface. Each animal was bandaged to prevent access to the wounds and individually housed. Rats were visually examined for signs of distress, discomfort, and weight loss. The wound was inspected for signs of implant rejection, inflammation, and peri-wound erythema. No evidence of inflammation or erythema was present for any of the wounds.

Tissue was bisected and on-half fixed overnight in 10% formalin prior to embedding a paraffin wax and 5 μm histological sections taken. All sections were stained with Haemataoxylin & Eosin (see below). One-half of the biopsy was snap-frozen in liquid nitrogen and stored at -20C.

Haematoxylin and Eosin staining

The paraffin wax sections were treated at room temperature as follows:

1. Immersed in filtered Harris' Haematoxylin for 3 minutes
2. Washed in running tap water for 10 minutes
3. Dipped in Eosin for 30 seconds
4. Rinsed in tap water and dehydrated through 50%, 70%, 90% and 100% alcohol.
5. Dipped in xylene and mounted under coverslips in practamount

Quantitation of the healing process was made at day 6 post-wounding microscopically by image analysis, determining the cross-sectional wound area, wound widths, cell numbers pet unit area (mm^2) and degree of epithelialization (measured as a percentage of new epithelium as a function of wound width).

Histological examination of the tissue included:

- Incorporation of the gel into surrounding dermal tissue
- Would area (histological cross-section at day 6 post-wounding) using Image Pro Plus (Media Cybemetics, Silver Spring, MD, USA) image analysis system
- Cell infitrate quantified using image analysis
- Degree of re-epithelialization (as a function of histological wound width)

Results of the rat dermal excisional wound trials:

Macroscopic appearance

No adverse effects to animal welfare were noted and there was no evidence of any wound causing irritation. There was no evidence of local erythema, infection nor inflammation at the wound site. Figures 12 and 13 of the accompanying drawings show microscopic cross-sectional images (x 5 and x 20 mag) of an unmanipulated wound (a) and a wound treated with a modified procollagen pNdec™ according to the present invention (b). The modified procollagen gel clearly integrates within the wound and appears to promote healing.

Histological Appearance

The cell numbers and wound widths are shown in Tables 2 and 3 below. The decorin gels had become incorporated into the edges of the wound. There were no differences in wound areas (cross-sectional) and % epithelialization at day 6 post-wounding compared to the unmanipulated wounds.

Table 2

Mouse Strain	Cell Numbers	Area (mm2)	Cells/mm2
1	1371	0.43	3188
1b	1276	0.43	2967
2	1726	0.35	4931
2b	1047	0.43	2435
3	1895	0.43	4406
3b	2687	0.43	6249
4	1865	0.43	4337
4b	2500	0.43	5814
5	1840	0.43	4279
5b	2698	0.43	6274

1 and 1b etc; are different areas of the same wound

Table 3

Rat Strain	Wound Width	% Re epithelialization	Wound Area
1	5.11	44	3.45
2	4.72	88	2.66
3	2.96	72	2.65
4	2.28	100	1.79
5	2.89	25	2.78

Cell numbers were similar between groups (cells per mean area), however the pNdec™ gels appeared to exhibit increased numbers of cells morphologically resembling fibroblasts. In addition, the pNdec™ gels showed increased levels of angiogenesis. Figures 14, 15 and 16 are block diagrams illustrating the wound area, % epithelialization and cell numbers/mm^2 6 days post wounding for unmanipulated wounds (UNM) and wounds treated with pNdec™ according to the invention (DEC).

The ability of the modified collagen gels according to the invention to integrate within the host tissue with minimal shrinkage indicates that the gels will be suitable for other applications in addition to wound healing, such as tissue reconstruction and cosmetic surgery.

EXAMPLE 10: Manufacture of a collagen gel according to the fifth aspect of the invention comprising a collagen polymer according to the fourth aspect of the invention that resists gel contraction by human skin fibroblasts.

Petri dishes (35 mm diameter) were used as a mould into which collagen gels were cast.

Type I bovine collagen (obtained from Vitrogen -Neutacon BV, The Netherlands) was used to prepare control gels. 8 ml Vitrogen collagen (3 mg/ml) was mixed with 1 ml of 10X Dulbecco's modified Eagle's medium (containing penicillin and streptomycin, 10% foetal calf serum and 1% L-glutamine) and 1 ml of 0.1 M NaOH. The pH was adjusted to 7.4. The collagen solution was poured into the Petri dishes (1.5 ml per dish) and heated to 37°C.

In test samples dec-proα1(III) or purified decorin were also added separately to the Vitrogen collagen solution prior to gel formation. The final amount of dec-proα1(III) in the polymerised collagen gel was 22.5 μg. The final amount of decorin in the polymerised collagen gel was 3.2 μg.

After 3 hours the gels had 'set' to a firm consistency. The gels were scored around their edges to release the gel from the plastic surface of the Petri dish.

One ml of a suspension of human skin fibroblasts (passage 10, 50,000 cells/ml) was added onto the top surface of each gel. The dimensions of the gel were recorded photographically every 48 hours. Culture medium (DMEM containing penicillin and streptomycin, 10% foetal calf serum and 1% L-glutamine) was replaced every 48 hours.

One example is shown in Figure 17 in which the left hand petri dishes were treated with decorin (N3), the middle petri dishes contained gels comprising dec-proα1(III) (D3) and the right hand petri dishes were control dishes in which contraction was allowed to occur without any treatments (C3).

The fact that gels comprising dec-proα1(III) are able to resist contraction illustrates that collagen matrices according to the present invention will be particularly useful for covering (artificial skins) or packing wounds; treating fibrotic conditions and also in cosmetic applications (e.g collagen injections which resist contraction).

Example 11: Manufacture of a collagen gel according to the Invention and an investigation into its resistance to gel contraction.

Collagen – pNdec gels were prepared using 1mg/ml rat type 1 collagen and 360 mg/ml pNdec. Gels were seeded with 50,000 human dermal fibroblasts and the diameter of the gels was determined over a 150 hour time period.

Figures 18a to 18b illustrate the results of a collagen control gel, after 2, 4 and 70 hours respectively. Figures 19a to 19c illustrate the results of a gel having 360 μg/ml pNdec in the gel. Figure 20 is a graph demonstrating collagen gel contraction in the presence absence of pNdec.

Analysis of the data showed that the gels containing the modified procollagen pNdec of the present invention were refractory to gel contraction during 70 hours which was markedly different to the collagen gel controls that experienced significant contraction at 70 hours. However, after 150 hours the pNdec-containing gels had contracted to the same size as gels in control samples.

EXAMPLE 12: Manufacture of a wound dressing incorporating a collagen matrix according to a fifth aspect of the invention.

A collagen matrix/gel according to a fifth aspect of the present invention was applied to a Combiderm N dressing. Figures 21 and 22 illustrate the wet gel 12 applied to a dressing 14 that is secured to an adhesive strip 16. The position of the gel is illustrated by dotted lines in the Figures. The matrix was then dehydrated before being applied to a wound with the matrix on the dressing. Figures 23 and 24 show the dehydrated gel 12 attached to the dressing 72 hours after application. This method of applying the matrix/gel to a wound was found to work well, avoiding the need to handle the gel, ensuring the dressing and gel remain clean and sterile and facilitating handling of the gel by the clinician.

CLAIMS

1 A modified pro-α chain comprising a triple helical forming domain linked to at least one N-terminal domain characterised in that the N-terminal domain contains a polypeptide sequence from at least part of a proteoglycan protein core.

2 A modified pro-α chain as claimed in claim 1 wherein the triple helical forming domain is from a fibrillar forming pro-α chain.

3 A modified pro-α chain as claimed in claim 2 wherein the triple helical forming domain is from a type I, II, III, V or XI pro-α chain.

4 A modified pro-α chain as claimed in claim 3 wherein the triple helical forming domain is from a proα1 (III) chain.

5 A modified pro-α chain as claimed in any one of claims 1 to 4 wherein the N-terminal domain comprises a whole proteoglycan molecule.

6 A modified pro-α chain as claimed in claim 5 wherein the N-terminal domain comprises decorin, biglycan, fibromodulin, lumican or functional derivatives thereof.

7 A modified pro-α chain as claimed in any one of claims 1 to 6 wherein the N-terminal domain comprises at least one leucine-rich repeat of 20-24 amino acids found in the decorin family of proteoglycans.

8 A modified pro-α chain as claimed in any one of claims 1 to 6 wherein the N-terminal domain comprises betaglycan or functional derivatives thereof.

9 A modified pro-α chain as claimed in any one of claims 1 to 8 wherein a N-proteinase cleavage site associated with the N-terminal propeptide domain is modified such as to alter the domain's susceptibility to cleavage.

10 A modified pro-α chain as claimed in claim 9 wherein the N-proteinase cleavage site is modified such that the domain may not be cleaved.

11 A modified pro-α chain as claimed in claim 10 wherein a region between the helical forming domain and the N-propeptide forming domain of the pro-α chain is modified to confer resistance to N-proteinases.

12 A modified pro-α chain as claimed in claim 11 wherein Pro-Gln in the region is altered to Leu-Pro.

13 A modified pro-α chain as claimed in claim 8 wherein the N-terminal domain contains the amino acids of SEQ ID NO: 9.

14 A DNA molecule encoding modified pro-α chains as defined by any one of claims 1 to 13.

15 A DNA molecule encoding modified pro-α chains as claimed in claim 14 characterised in that the molecule includes the bases of SEQ ID NO: 1.

16 A DNA molecule encoding modified pro-α chains as claimed in claim 14 characterised in that the molecule includes the bases of SEQ ID NO: 8.

17 A procollagen molecule comprising a trimer of pro-α chains characterised in that at least one of the pro-α chains is a modified pro-α chain as defined by any one of claims 1 to 13.

18 A procollagen molecule as claimed in claim 17 characterised in that the molecule is loaded with a growth factor for release at a target site.

19 A procollagen molecule as claimed in claim 18 wherein the growth factor is TGF-β1.

20 A procollagen molecule as claimed in claim 18 wherein the growth factor is TGF-β3.

21 A procollagen molecule as claimed in any one of claims 17 to 20 wherein the C-terminal domain of the molecule is removed.

22 A collagen polymer comprising collagen monomers wherein at least some of the collagen monomers contained therein have retained N-propeptides characterised in that at least some of the retained N-propepetides contain a polypeptide sequence from at least part of a proteoglycan protein core.

23 A collagen polymer as claimed in claim 22 wherein the collagen monomers having retained propeptide domains are derived from procollagen molecules as defined by any one of claims 17 to 21.

24 A shrinkage resistant collagen matrix comprising collagen monomers characterised in that at least some of the monomers have modified N-terminals domains to provide a matrix that is resistant to contraction.

25 A collagen matrix as claimed in claim 24 wherein at least some of the collagen monomers have an N-terminal domain containing leucine rich repeat sequences.

26 A collagen matrix as claimed in claim 25 wherein at least some of the collagen monomers have an N-terminal domain containing a polypeptide sequence from a proteoglycan.

27 A collagen matrix as claimed in claim 26 wherein the N-terminal domain comprises decorin, biglycan, fibromodulin, lumican or functional derivatives thereof.

28 A collagen matrix as claimed in claim 27 wherein the N-terminal domain comprises at least one leucine-rich repeat of 20-24 amino acids found in the decorin family of proteoglycans.

29 A collagen matrix as claimed in claim 28 wherein up to 50% of the matrix comprises collagen having said modified N-terminal domains.

30 A collagen matrix as claimed in claim 24 wherein the collagen monomers having modified propeptide domains are derived from procollagen molecules as defined by any one of claims 17 to 21.

31 A dressing comprising collagen polymers as defined by claim 22 or 23 or a collagen matrix as defined by any one of claims 24 to 30.

32 The use of a modified pro-α chain, procollagen molecule, polymer, matrix or dressing according to any one of the preceding claims for the treatment of medical conditions.

33 The use of a modified pro-α chain, procollagen molecule, polymer, matrix or dressing according to any one of claims 1 to 31 for the manufacture of a medicament for use in the treatment of wounds or fibrotic disorders.

34 A method of treating a wound or fibrotic disorder comprising administering to a subject in need of such treatment a therapeutically effective amount of a modified pro-α chain, procollagen molecule, polymer, matrix or dressing according to any one of claims 1 to 31.

35 The use of a modified pro-α chain, procollagen molecule, polymer or matrix according to any one of claims 1 to 30 for coating medical products or devices.

36 The use of a modified pro-α chain, procollagen molecule, polymer or matrix as claimed in claim 35 for coating a catheter.

37 An artificial skin/tissue comprising a collagen matrix according to any one of claims 23 to 30.

38 A body implant comprising a collagen matrix according to any one of claims 23 to 30.

39 The use of a collagen matrix, artificial skin/tissue or a body implant according to any one of claims 23 to 30, claim 37 or claim 38 for the treatment of medical conditions.

40 The use of a collagen matrix, artificial skin/tissue or a body implant according to any one of claims 23 to 30, claim 37 or claim 38 in a cosmetic treatment.

41 The use of a collagen matrix, artificial skin/tissue or a body implant as claimed in claim 40 as an advanced filler material in facial asthetic procedures.

42 A delivery system for use in gene therapy technique, said delivery system comprising a DNA molecule according to any one of claims 14 to 16 which is capable of being transcribed to lead the expression of the modified pro-α chain at a wound site or site of fibrosis.

43 The use of a delivery system as defined in claim 42 in the manufacture of a medicament for treating wounds or fibrotic disorders.

44 A method of treating a wound or fibrotic condition comprising administering to a patient in need of treatment a therapeutic dose of a delivery system as defined in claim 42.

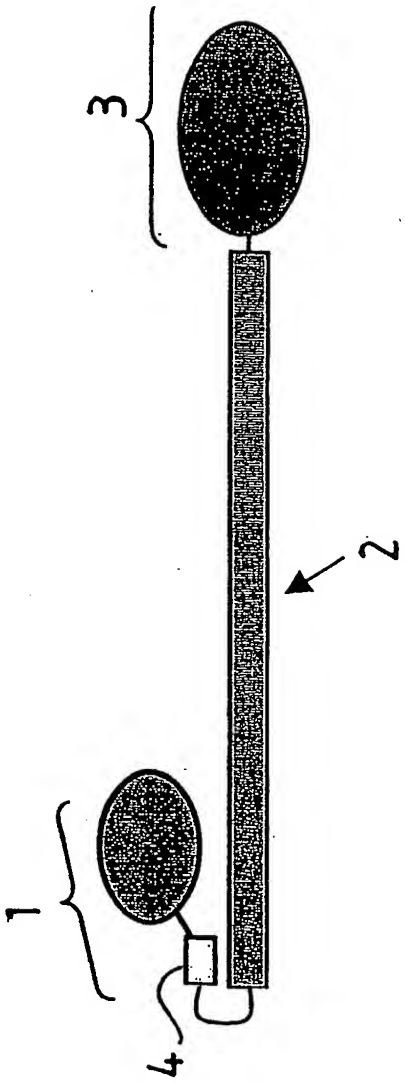

FIG.1

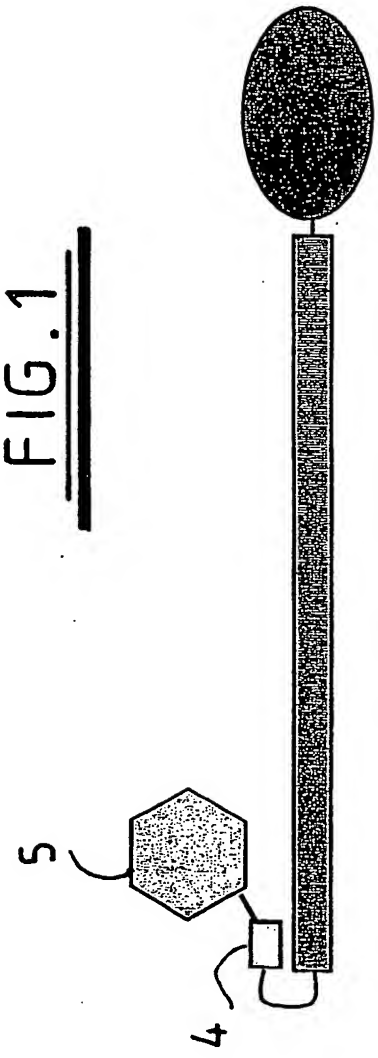

FIG.2

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gcccctgggt tccgaggacc tgctggacca aatggcatcc caggagaaaa gggtcctgct
ggagagcgtg gtgctccagg ccctgcaggg cccagaggag ctgctggaga acctggcaga
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agtgatggga aaccagggcc tcccggaagt caaggagaaa gtggtcgacc aggtcctcct
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gatggtgctc ctggtaagaa tggagaacga ggtggccctg gaggacctgg ccctcagggt
cctcctggaa agaatggtga aactggacct caaggacccc cagggcctac tgggcctggt
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ggtggtcctc caggagaaaa tggaaaacct ggggaaccag gtccaaaggg tgatgccggt
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…ggcggcccag gtgctgatgg tgtcccaggg aaagatggcc caaggggtcc tactggtcct
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cttccaggta tagctggacc tcgtggtagc cctggtgaga gaggtgaaac tggccctcca
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FIG. 3

agaggggctc cgggtgagaa aggtgaagga ggccctcctg gagttgcagg acccccctgga
ggttctggac ctgctggtcc tcctggtccc caaggtgtca aaggtgaacg tggcagtcct
ggtggacctg gtgctgctgg cttccctggt gctcgtggtc ttcctggtcc tcctggtagt
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cttgggattg ctgggatcac tggagcacgg ggtcttgcag gaccaccagg catgccaggt
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gggattggag gtgaaaaagc tggcggtttt gccccgtatt atggagatga accaatggat
ttcaaaatca acaccgatga gattatgact tcactcaagt ctgttaatgg acaaatagaa
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ttggatgcta tcaaggtatt ctgtaatatg gaaactgggg aaacatgcat aagtgccaat
cctttgaatg ttccacggaa acactggtgg acagattcta gtgctgagaa gaaacacgtt
tggtttggag agtccatgga tggtggtttt cagtttagct acggcaatcc tgaacttcct
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aagaaggccc tgaagctgat ggggtcaaat gaaggtgaat tcaaggctga aggaaatagc
aaattcacct acacagttct ggaggatggt tgcacgaaac acactgggga atggagcaaa
acagtctttg aatatcgaac acgcaaggct gtgagactac ctattgtaga tattgcaccc
tatgacattg gtggtcctga tcaagaattt ggtgtggacg ttggccctgt ttgcttttta
taa

(Fig. 3 continued)

```
M K A T I I L L L L A Q V S W A G P F Q Q R G L F D F M L E D
E A S G I G P E V P D D D R D F E P S L G P V C P F R C Q C H L
R V V Q C S D L G L D K V P K D L P P D T T L L D L Q N N K I
T E I K D G D F K N L K N L H A L I L V N N K I S K V S P G A
F T P L V K L E R L Y L S K N Q L K E L P E K M P K T L Q E L
R A H E N E I T K V R K V T F N G L N Q M I V I E L G T N P L
K S S G I E N G A F Q G M K K L S Y I R I A D T N I T S I P Q
G L P P S L T E L H L D G N K I S R V D A A S L K G L N N L A
K L G L S F N S I S A V D N G S L A N T P H L R E L H L D N N
K L T R V P G G L A E H K Y I Q V V Y L H N N N I S V V G S S
D F C P P G H N T K K A S Y S G V S L F S N P V Q Y W E I Q P
S T F R C V Y V R S A I Q L G N Y K N G Q G P Q G P K G D P G
P P G I P G R N G D P G I P G Q P G S P G S P G P P G I C E S
C P T G P Q N Y S P Q Y D S Y D V K S G V A V G G L A G Y P G
P A G P P G P P G P P G T S G H P G S P G S P G Y Q G P P G E
P G Q A G P S G P P G P P G A I G P S G P A G K D G E S G R P
G R P G E R G L P G P P G I K G P A G I P G F P G M K G H R G
F D G R N G E K G E T G A P G L K G E N G L P G E N G A P G P
M G P R G A P G E R G R P G L P G A A G A R G N D G A R G S D
G Q P G P P G P P G T A G F P G S P G A K G E V G P A G S P G
S N G A P G Q R G E P G P Q G H A G A Q G P P G P P G I N G S
P G G K G E M G P A G I P G A P G L M G A R G P P G P A G A N
G A P G L R G G A G E P G K N G A K G E P G P R G E R G E A G
I P G V P G A K G E D G K D G S P G E P G A N G L P G A A G E
R G A P G F R G P A G P N G I P G E K G P A G E R G A P G P A
G P R G A A G E P G R D G V P G G P G M R G M P G S P G G P G
S D G K P G P P G S Q G E S G R P G P P G P S G P R G Q P G V
M G F P G P K G N D G A P G K N G E R G G P G G P G P Q G P P
G K N G E T G P Q G P P G P T G P G G D K G D T G P P G P Q G
L Q G L P G T G G P P G E N G K P G E P G P K G D A G A P G A
P G G K G D A G A P G E R G P P G L A G A P G L R G G A G P P
G P E G G K G A A G P P G P P G A A G T P G L Q G M P G E R G
G L G S P G P K G D K G E P G G P G A D G V P G K D G P R G P
T G P I G P P G P A G Q P G D K G E G G A P G L P G I A G P R
G S P G E R G E T G P P G P A G F P G A P G Q N G E P G G K G
E R G A P G E K G E G G P P G V A G P P G G S G P A G P P G P
Q G V K G E R G S P G G P G A A G F P G A R G L P G P P G S N
G N P G P P G P S G S P G K D G P P G P A G N T G A P G S P G
V S G P K G D A G Q P G E K G S P G A Q G P P G A P G P L G I
A G I T G A R G L A G P P G M P G P R G S P G P Q G V K G E S
G K P G A N G L S G E R G P P G P Q G L P G L A G T A G E P G
R D G N P G S D G L P G R D G S P G G K G D R G E N G S P G A
P G A P G H P G P P G P V G P A G K S G D R G E S G P A G P A
G A P G P A G S R G A P G P Q G P R G D K G E T G E R G A A G
I K G H R G F P G N P G A P G S P G P A G Q Q G A I G S P G P
A G P R G P V G P S G P P G K D G T S G H P G P I G P P G P R
G N R G E R G S E G S P G H P G Q P G P P G P P G A P G P C C
G G V G A A A I A G I G G E K A G G F A P Y Y G D E P M D F K
I N T D E I M T S L K S V N G Q I E S L I S P D G S R K N P A
R N C R D L K F C H P E L K S G E Y W V D P N Q G C K L D A I
K V F C N M E T G E T C I S A N P L N V P R K H W W T D S S A
```

FIG. 4

E K K H V W F G E S M D G G F Q F S Y G N P E L P E D V L D V
Q L A F L R L L S S R A S Q N I T Y H C K N S I A Y M D Q A S
G N V K K A L K L M G S N E G E F K A E G N S K F T Y T V L E
D G C T K H T G E W S K T V F E Y R T R K A V R L P I V D I A
P Y D I G G P D Q E F G V D V G P V C F L

(Fig. 4 continued)

Dec-proα1(III) Integra flask production course and CT/T digest.

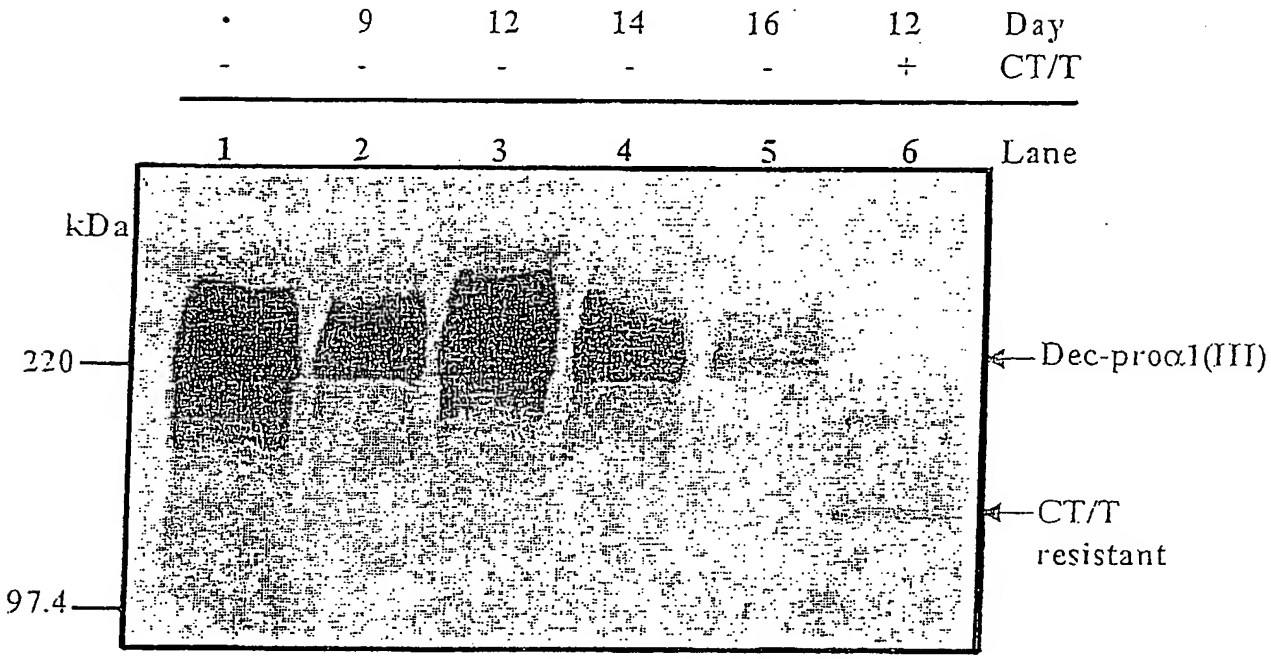


FIG. 5

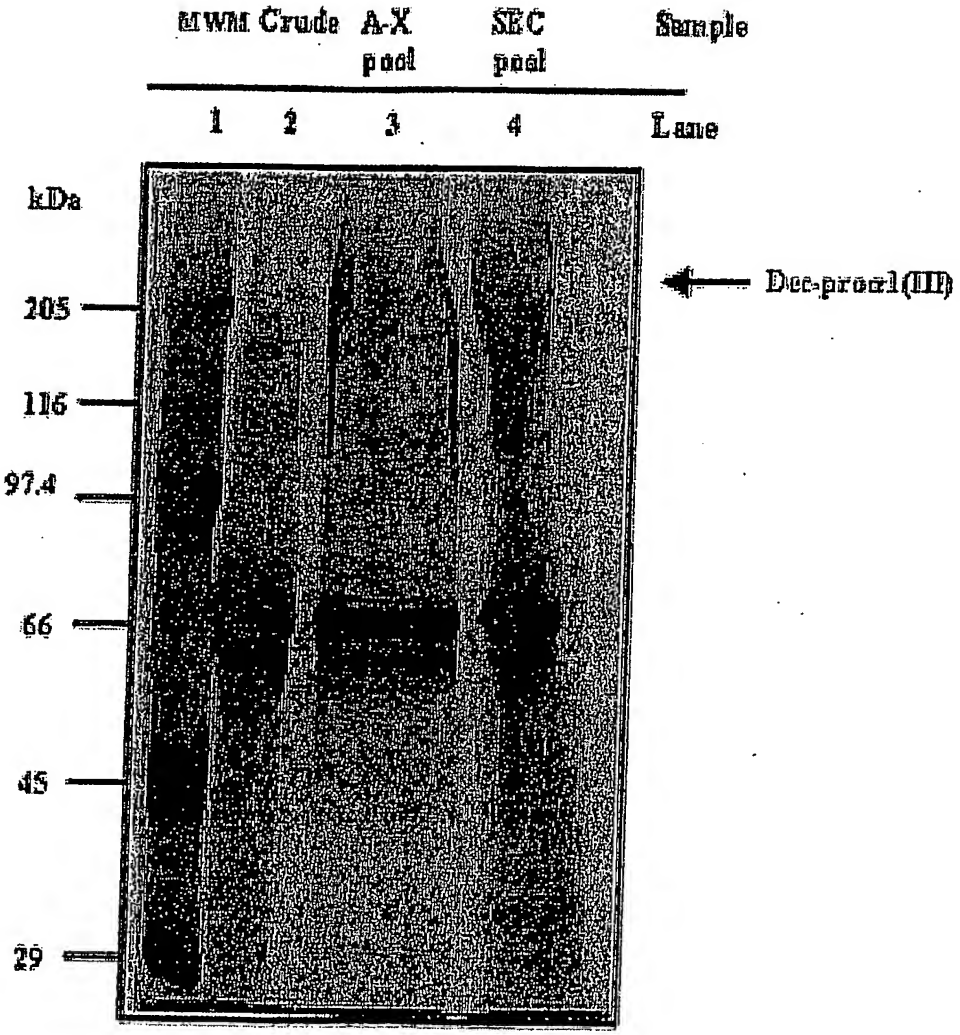

MWM
Crude
A-X pool
SEC pool
Sample
1
2
3
4
Lane
kDa
205
116
97.4
66
45
29
Dec-procol(III)

FIG. 6

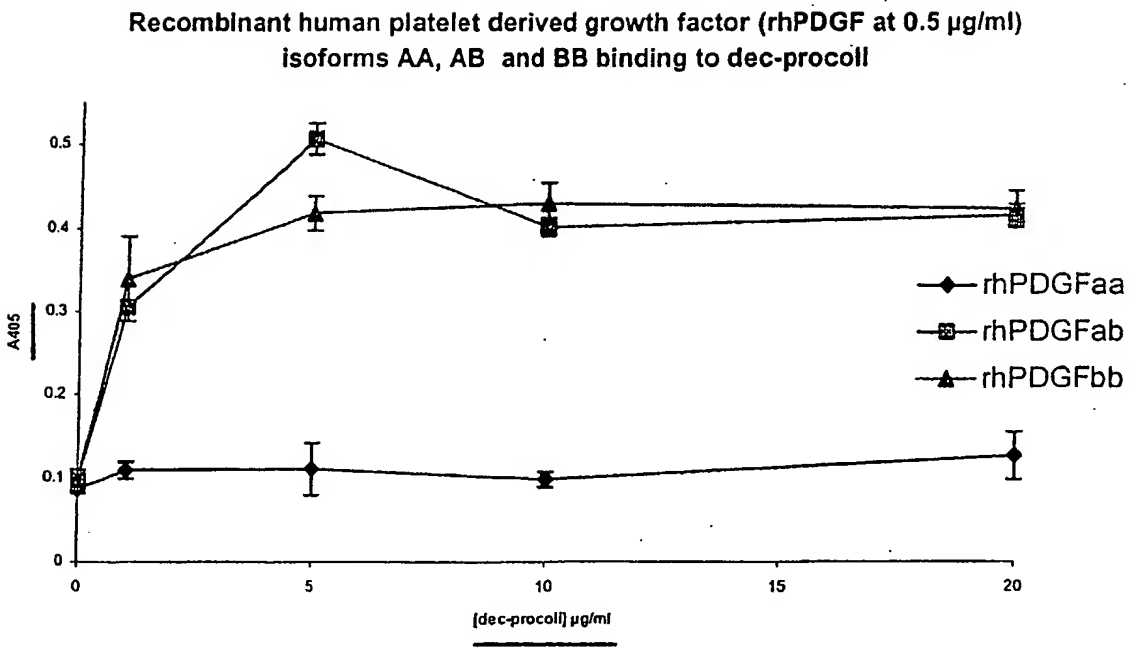

Recombinant human platelet derived growth factor (rhPDGF at 0.5 µg/ml) isoforms AA, AB and BB binding to dec-procoll
A405
0.5
0.4
0.3
0.2
0.1
0
0
5
10
15
20
[dec-procoll] µg/ml
rhPDGFaa
rhPDGFab
rhPDGFbb

FIG.7

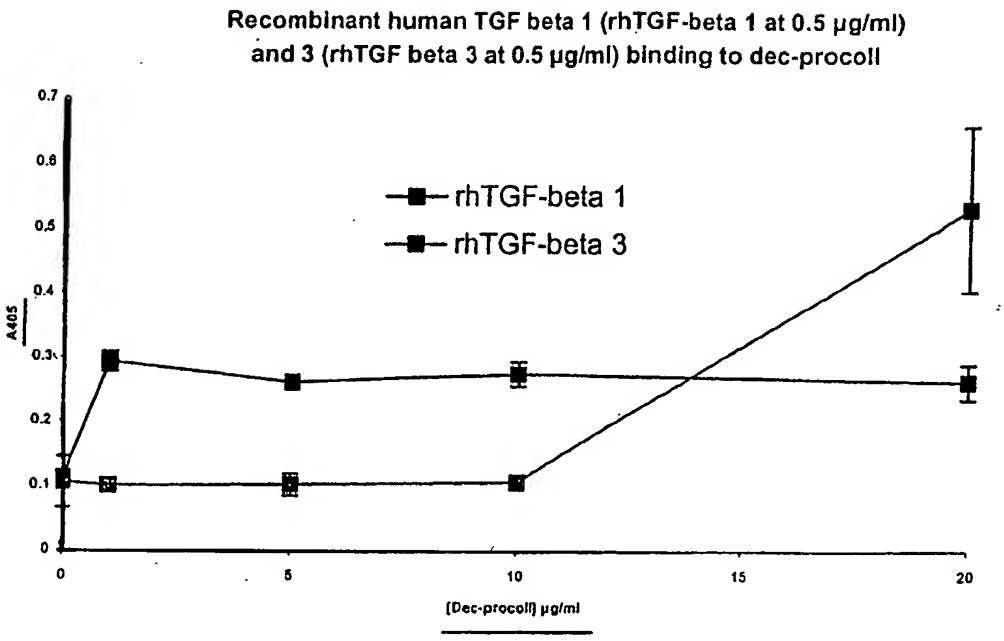
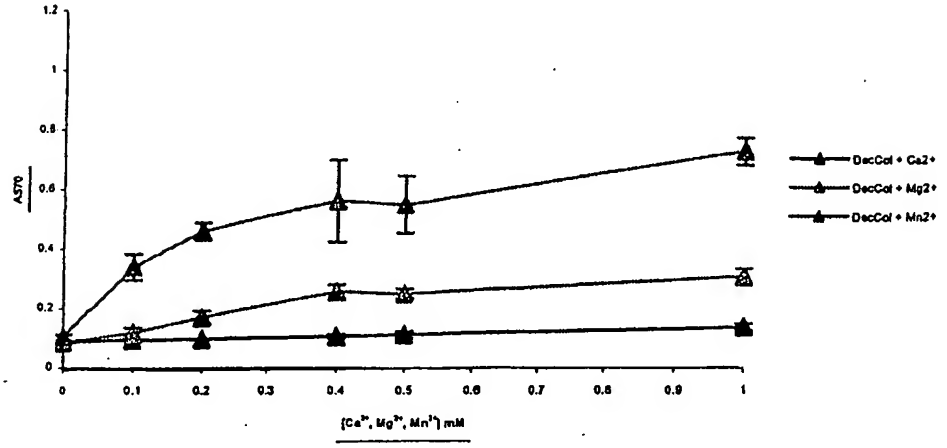

Recombinant human TGF beta 1 (rhTGF-beta 1 at 0.5 µg/ml)
and 3 (rhTGF beta 3 at 0.5 µg/ml) binding to dec-procoll
rhTGF-beta 1
rhTGF-beta 3
A405
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
5
10
15
20
[Dec-procoll] µg/ml

FIG. 8

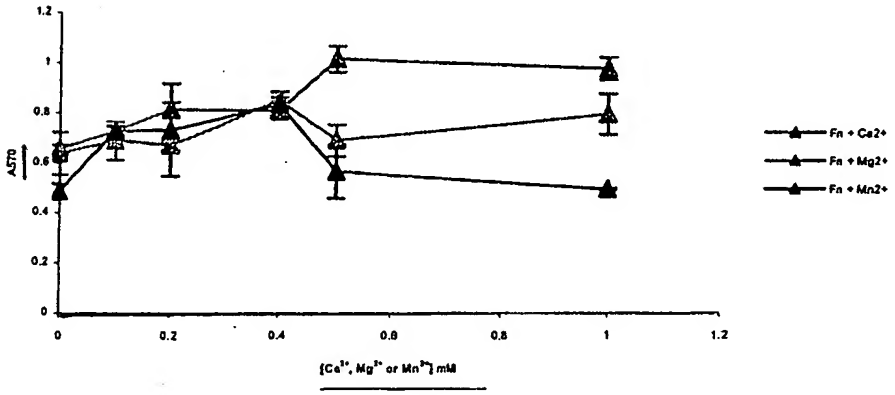
(A)

Human skin fibroblast attachment to dec-procollagen (20 µg/ml) in the presence of Ca2+, Mg2+ and Mn2+



(B)

Human skin fibroblast attachment to human fibronectin (20 µg/ml) in the presence of Ca^{2+}, Mg^{2+} and Mn^{2+}



(C)

Human skin fibroblast attachment to dec-procollagen and human fibronectin (Fn) in the presence of 1 mM $MnCl_2$

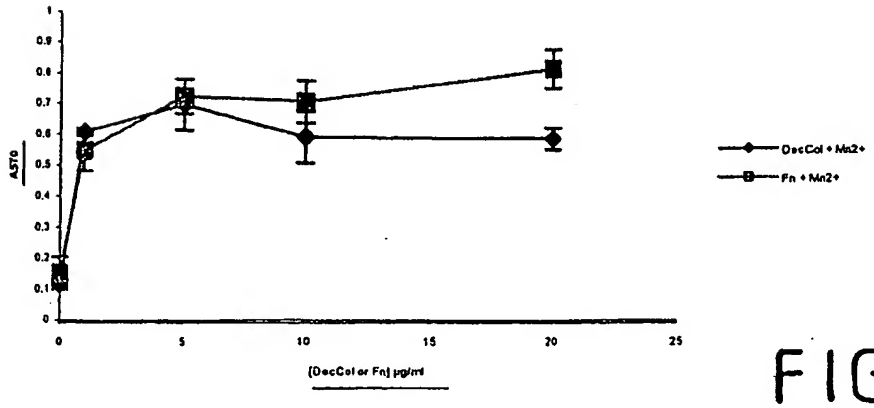
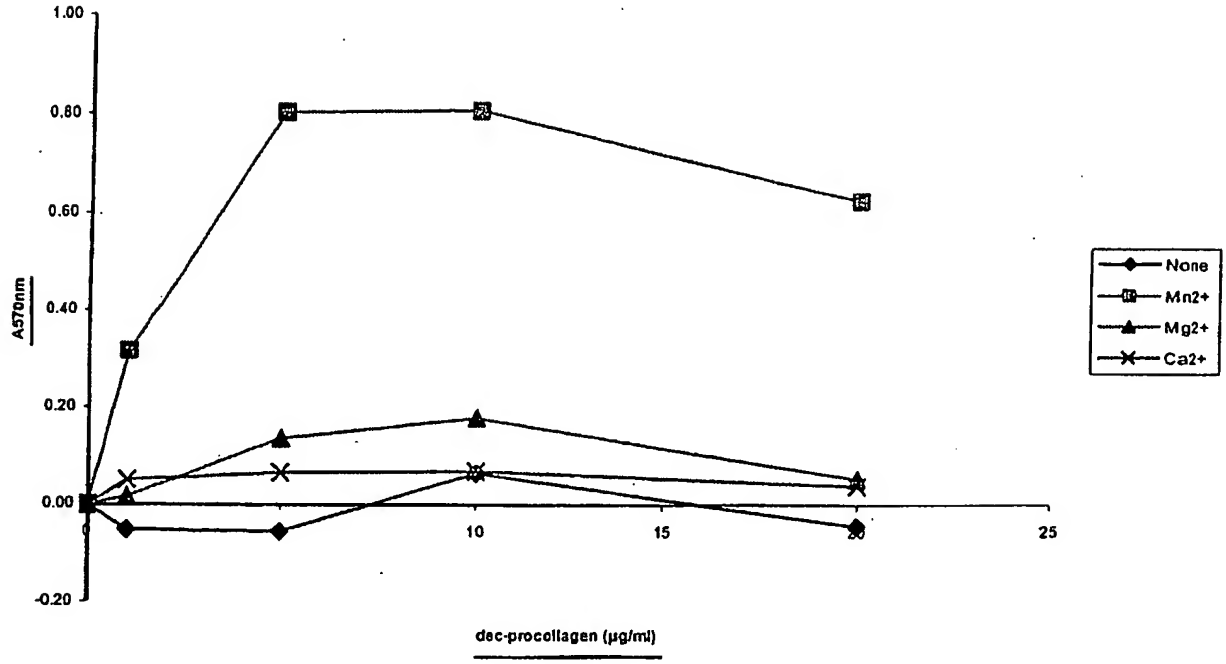


FIG. 9

(A).


1.00
0.80
0.60
0.40
0.20
0.00
-0.20
A570nm
0
10
15
20
25
dec-procollagen (µg/ml)
None
Mn2+
Mg2+
Ca2+

(B)

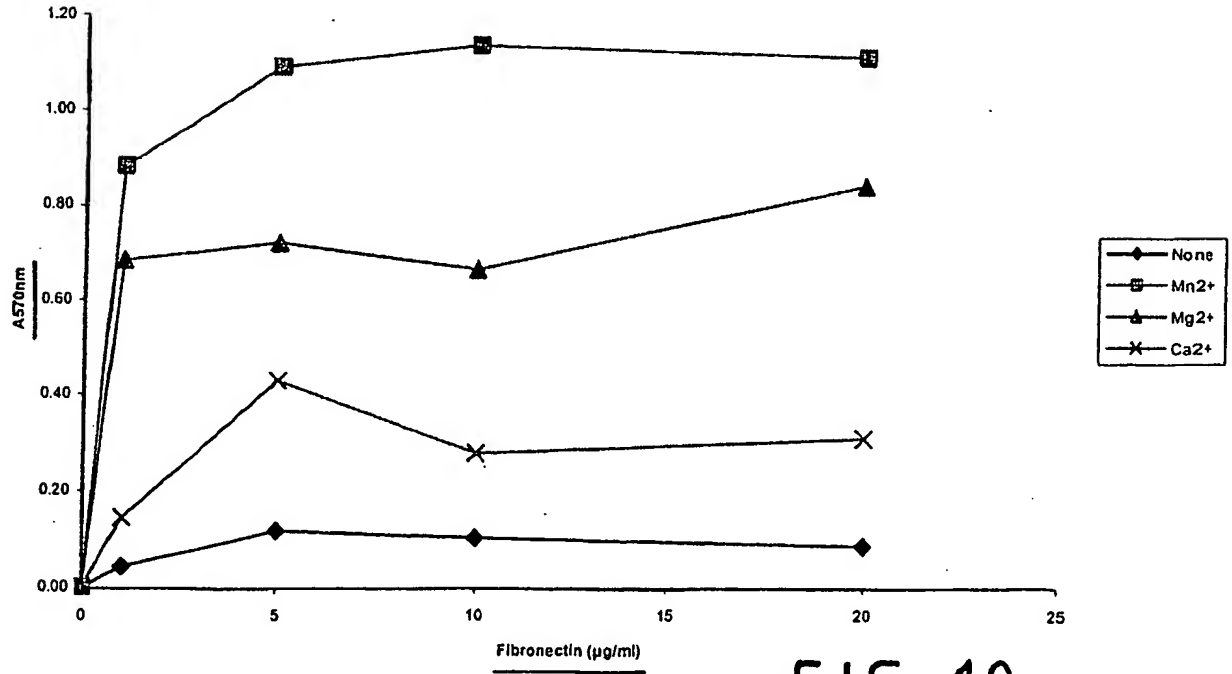

1.20
1.00
0.80
0.60
0.40
0.20
0.00
A570nm
0
5
10
15
20
25
Fibronectin (µg/ml)
None
Mn2+
Mg2+
Ca2+

FIG.10

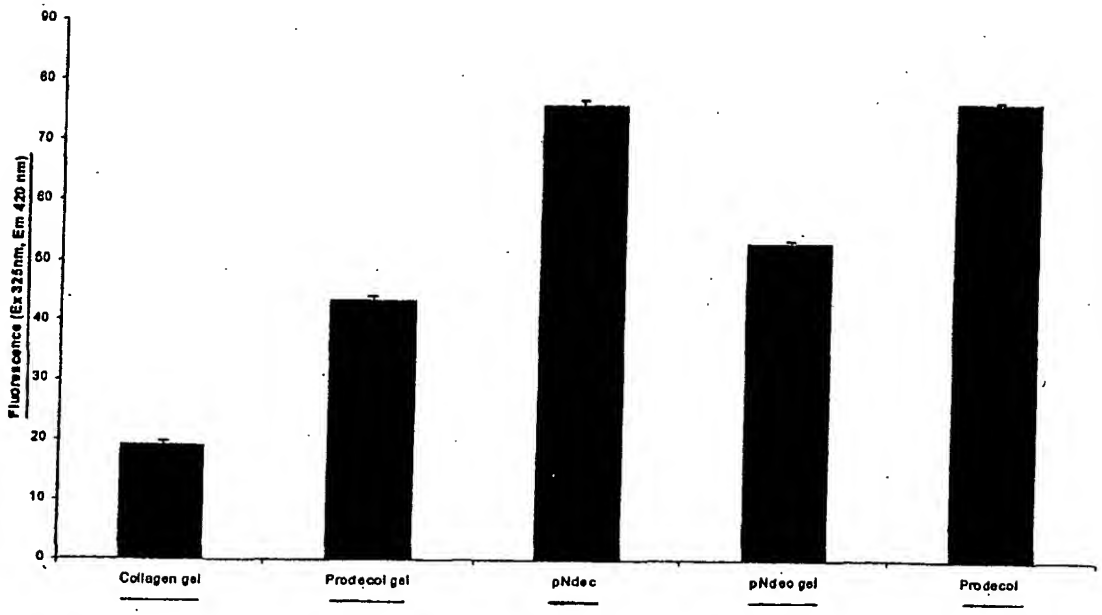

Fluorescence (Ex 325nm, Em 420 nm)
90
80
70
60
50
40
30
20
10
0
Collagen gel
Prodecol gel
pNdec
pNdeo gel
Prodecol

FIG. 11

(b)

(a)

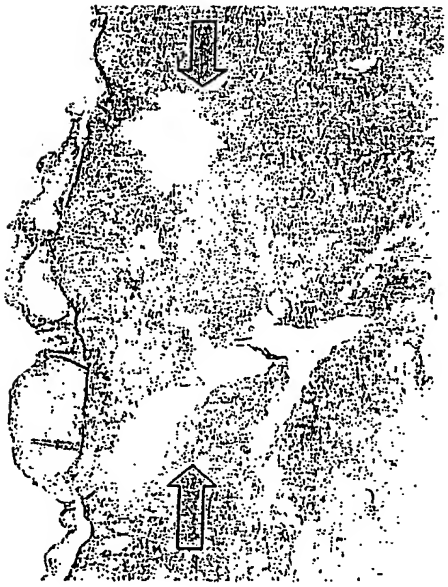


FIG. 12

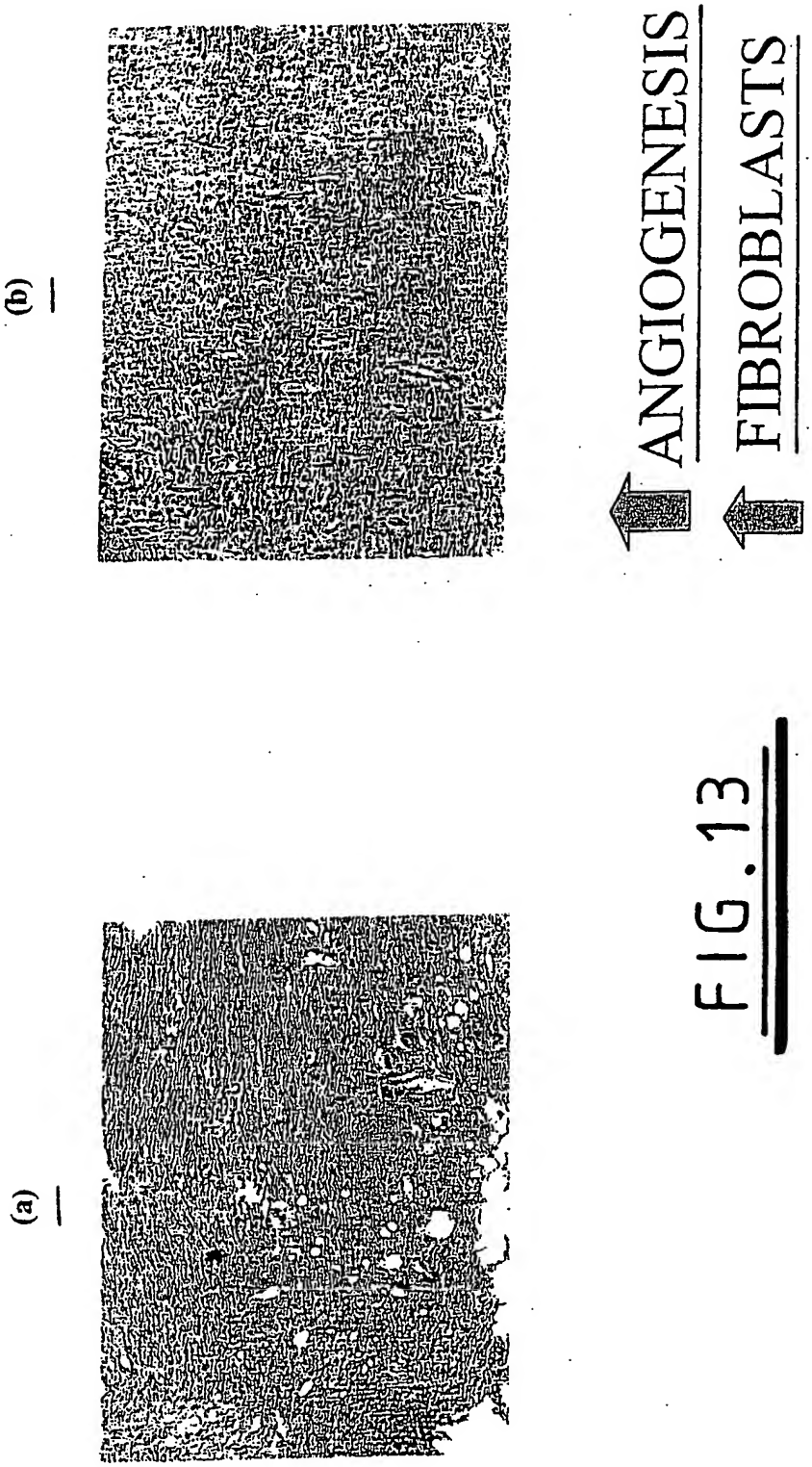


FIG. 13

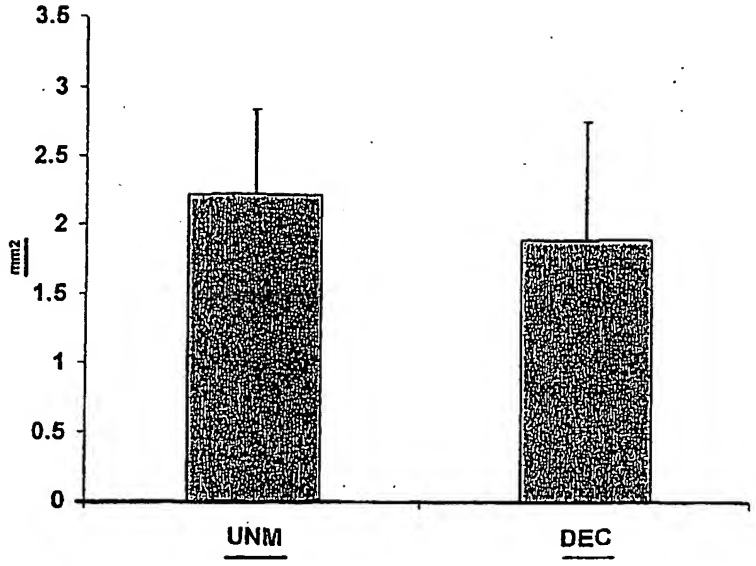


FIG. 14

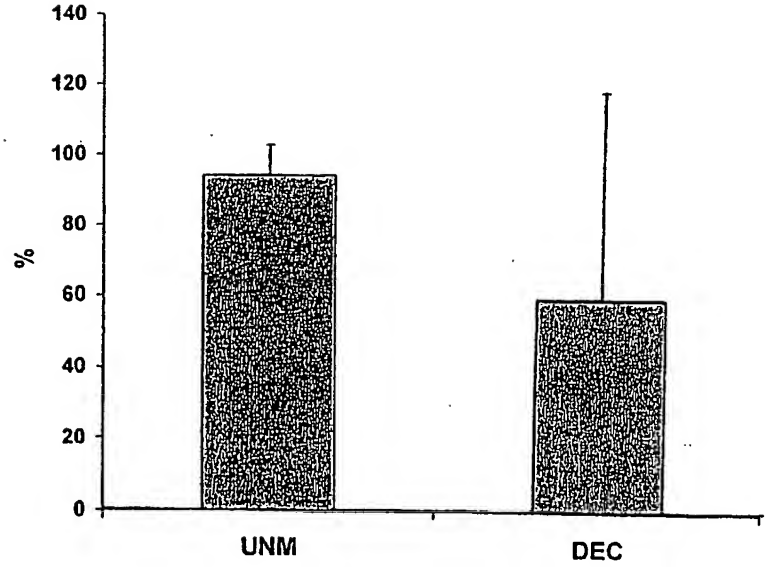


FIG. 15

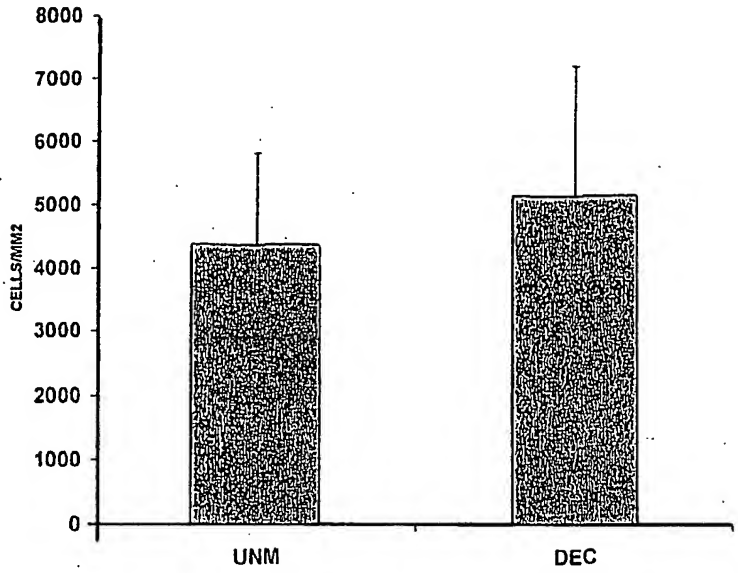
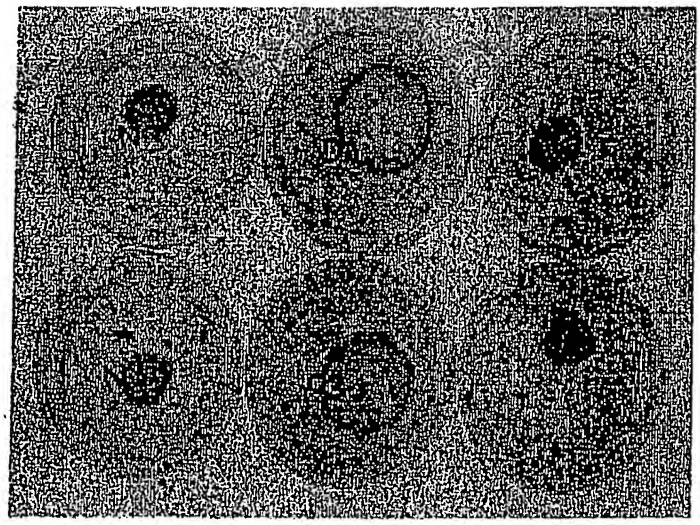
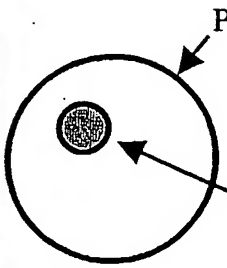


FIG.16





Contracted collagen gel in samples not containing dec-procollagen.

FIG.17

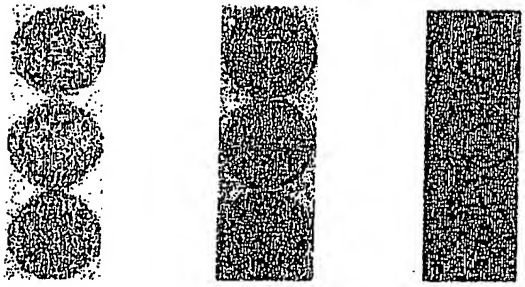


FIG.18a | FIG.18c

FIG.18b

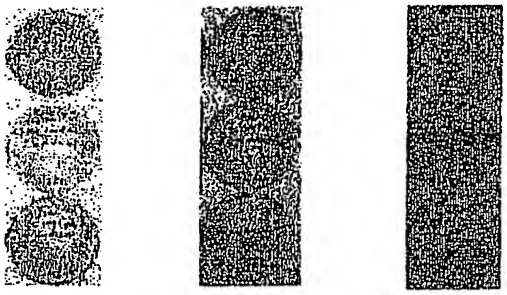


FIG.19a | FIG.19c

FIG.19b

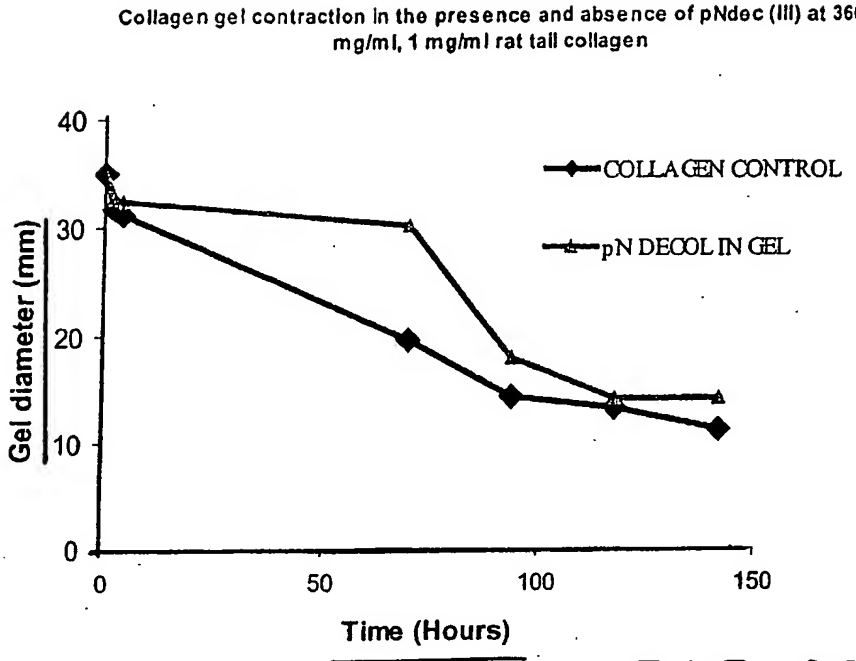

Collagen gel contraction in the presence and absence of pNdec (III) at 360 mg/ml, 1 mg/ml rat tail collagen
COLLAGEN CONTROL
pN DECOL IN GEL
Gel diameter (mm)
Time (Hours)
0
10
20
30
40
0
50
100
150

FIG.20

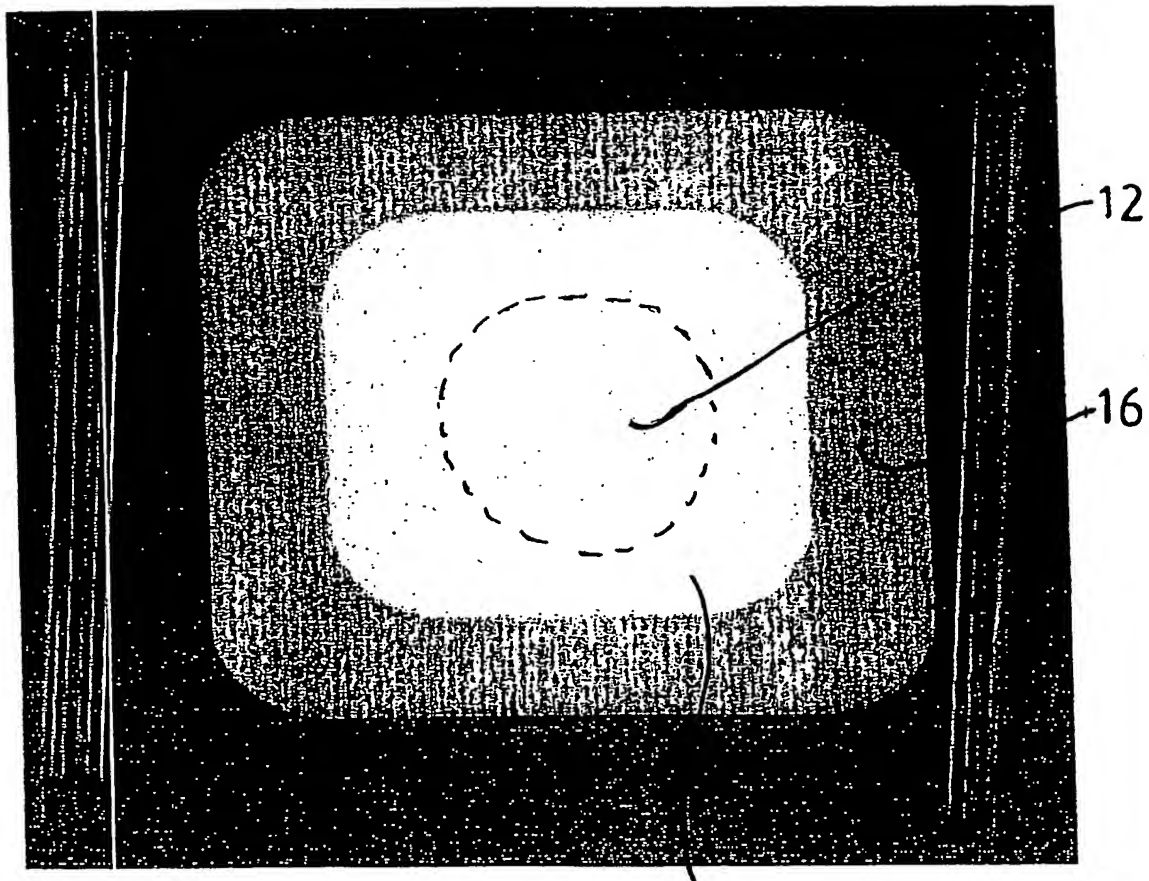

12
16
14

FIG.21

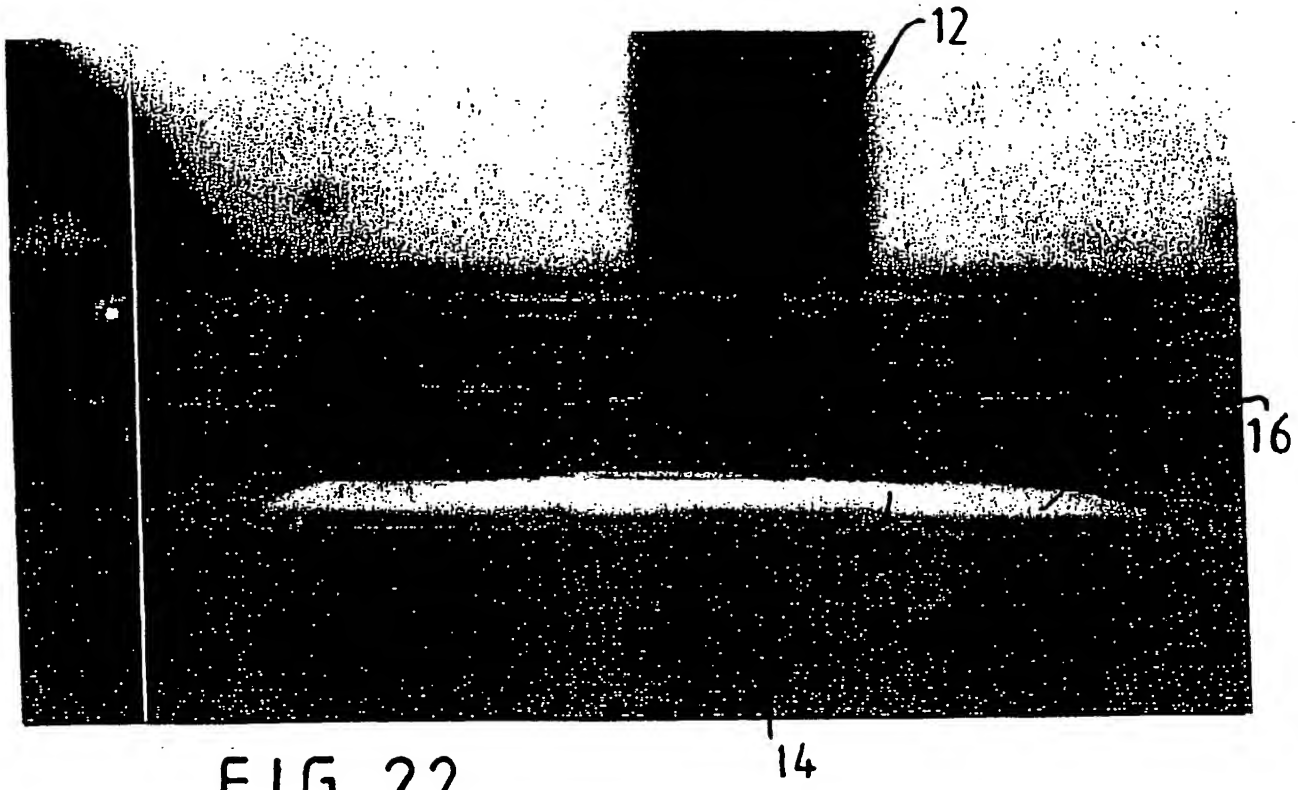


FIG. 22

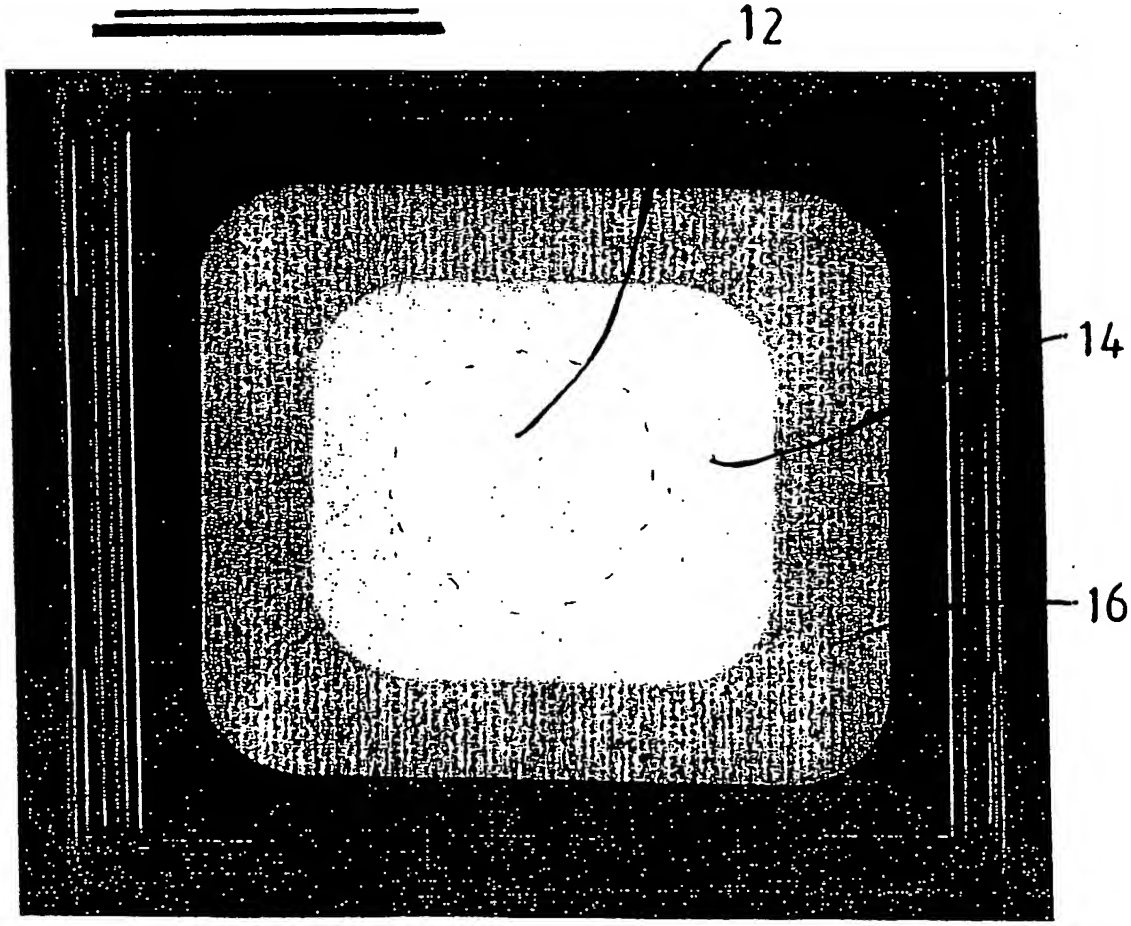


FIG. 23

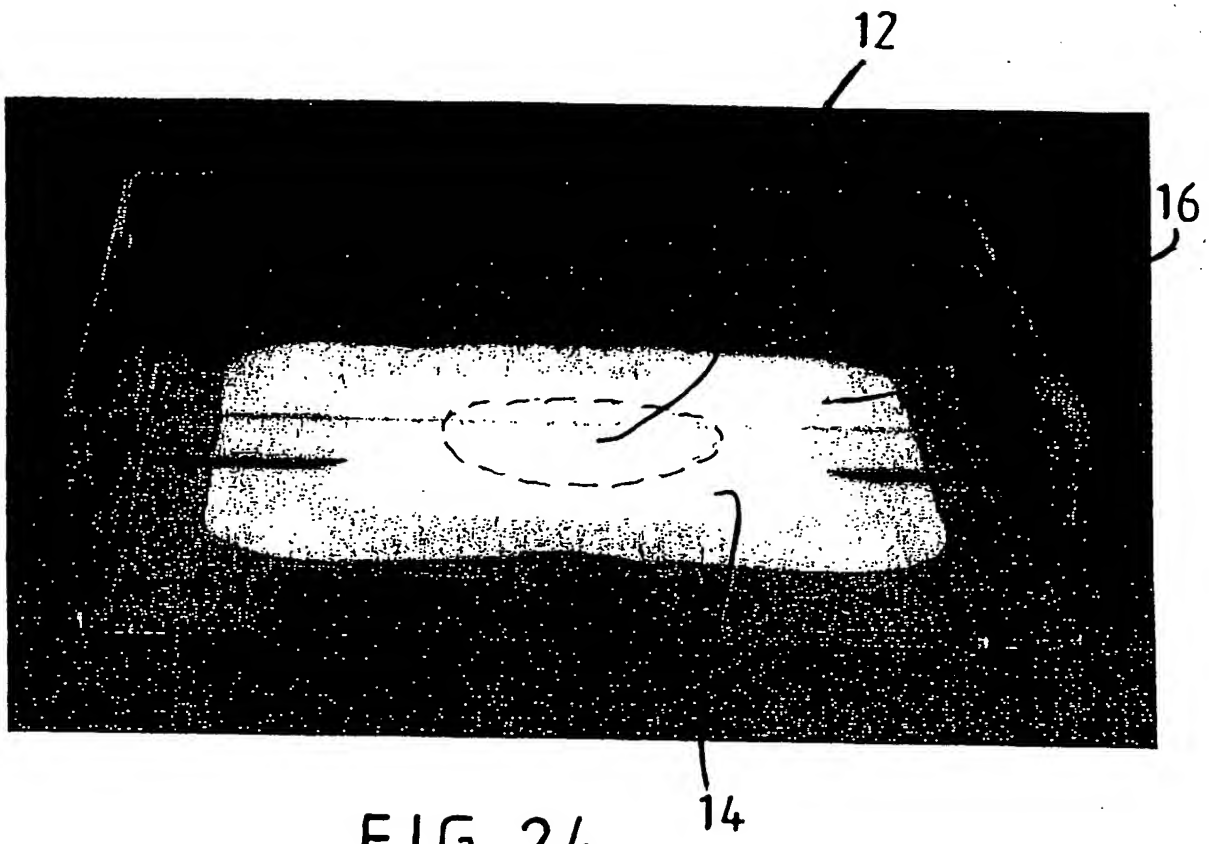


FIG. 24

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